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SOME FEATURES OF ATMOSPHERIC EDDIES WITH APPLICATIONS TO THE VIBRATIONS OF TRANSMISSION LINES¹

BY W. E. KNOWLES MIDDLETON²

Abstract

Some conclusions regarding atmospheric eddies, derived from the records of a pressure-tube anemograph at St. Hubert Airport near Montreal, are applied to the problem of the vibrations of transmission lines, furnishing a meteorological approach to the problem recently discussed by C. D. Niven.

It is found (1) that during glaze storms the wind at 60 ft. above ground is generally less gusty than the average; (2) that the mean duration of a gust varies inversely as the square root of the wind velocity, and is too large to resonate with the ordinarily observed period of "galloping"; and (3) that the steadiest winds invariably occur on nights of low wind velocity when a large negative lapse rate of temperature has been built up, leading to the small high-frequency vibrations due to eddies forming behind the cables.

The meteorological conditions accompanying a recent destructive case of "galloping" form the subject of part of the paper.

Introduction

C. D. Niven (8) has recently discussed from the experimental standpoint the problem of the various vibrations of transmission lines. This problem has of recent years proved of great practical importance to the engineering fraternity, especially where it concerns the vibrations of large amplitude, known as "galloping", "whipping", or "dancing". This part of the problem has been treated theoretically by DenHartog (4) and Varney (11), and from a general engineering standpoint by Archbold (1), Davison (2) and others. This paper is an attempt to contribute to the solution of the problem by setting forth some of the meteorological factors involved.

Data Available

The anemograph at St. Hubert Airport, near Montreal, gives a continuous record of wind velocity and direction at a height of 60 ft. above the ground. The normal record is on a time scale of 0.60 in. per hr., but a special quick-run drum is available, which has a time scale for the velocity of 7.2 in. per hr., making it possible to count the individual gusts. The normal records have been taken continuously from July 1930 until the present, and have

¹ Manuscript received January 9, 1933.

² Contribution from the Meteorological Service of Canada. Published by permission of the Director of the Meteorological Service of Canada.

³ Meteorologist, Meteorological Service of Canada.

yielded much information, shortly to be published, about the general *regime* of winds at St. Hubert. The quick-run records have been taken daily between 10h and 12h E.S.T. during the last four months.

Winds Associated with Glaze Storms

It has been suggested that "sleet storms"* may be associated with particularly gusty winds. As the gustiness of winds is largely influenced by convective processes, and as convection is greatest on bright sunshiny days, the suggestion seems *prima facie* unlikely. To test the matter, data for all the occasions during the winter of 1931-32 on which ice was reported to have formed on exposed objects at St. Hubert were examined, and the gustiness on each occasion compared with the mean gustiness for the same hour of the same month. The definition of gustiness adopted by the Meteorological Service of Canada is the one given by Dines (5), namely,

$$\text{Gustiness} = \frac{\text{max. vel.} - \text{min. vel.}}{\text{mean vel.}}$$

these quantities being taken over the entire hour.†

TABLE I
GUSTINESS IN GLAZE STORMS AT ST. HUBERT

Date	Hour ending	Gustiness		Remarks
		This hr.	Mean	
Dec. 11, 1931	16 h	0.80	0.82	One lull increased gustiness
Jan. 17, 1932	14	0.72	0.85	
Feb. 11	14	1.40	1.06	
Feb. 17	14	0.68	1.06	
Feb. 27	08	1.00	0.87	Anemograph doubtful
Mar. 22	14	0.25	0.53	
	16	0.33	0.52	
April 2	20	0.35	0.61	

It will be seen from Table I that only on two occasions was the gustiness during the glaze storm greater than the mean for the hour. Examination of the original records shows that on February 11 it was almost doubled by an isolated lull in the wind, and except for this would have been about 0.75. There is good reason to believe that on February 27 the anemograph was not functioning properly. In general, the results of this comparison support the inferences that would be drawn from general meteorological theory, which are further borne out by the large negative correlation coefficient (-0.65) at St. Hubert between mean monthly gustiness and mean monthly amount of cloud.

* The official meteorological definition of the word "sleet" on this continent is clear pellets of frozen rain. Ice accumulating on exposed objects is called "glaze". The engineering profession have in the past used "sleet" broadly to include this latter phenomenon, but the present tendency is to use the word "glaze" in conformity with North American meteorological usage.

† Since April 1932, the gustiness has been calculated from the records for ten minutes previous to each hour, instead of from the entire hour. This change does not affect the present results.

The gustiness calculated in this manner is only one of the many attributes of the wind; the above comparison by no means disposes of the possibility that the wind may have some peculiar feature during glaze storms, or even during some glaze storms and not others. More detailed examination of the wind structure is therefore necessary.

Duration of a Gust

It has been pointed out by Giblett and others (6) that the fluctuations in the direction and velocity of the wind are of three kinds. First there are the small rapid fluctuations with periods of the order of ten seconds, produced by the small eddies due to surface friction. Next, and superimposed on these, are the curious fluctuations with periods of the order of five minutes, for

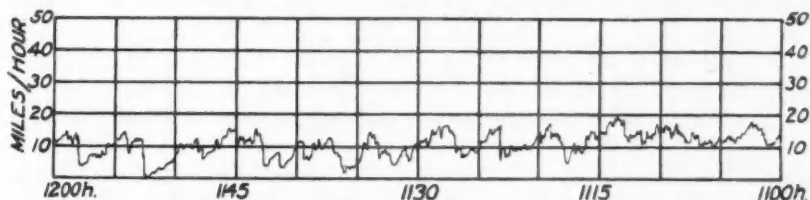


FIG. 1. Velocity of wind from 11h to 12h on May 10, 1932. Note that time scale runs from right to left.

which an ingenious theory has been proposed by Durst in the same paper (6). These two sets of fluctuations are well shown in the portion of the quick-run record of May 10, 1932, reproduced in Fig. 1. Thirdly, there are the major fluctuations of the order of a day or several days, due to the passage of barometric pressure-systems across the station. Of these three, only the first kind touches the present problem.

To arrive at the duration of a gust, or rather of a gust-and-lull, the quick-run records were divided into 5-min. intervals, and these into $2\frac{1}{2}$ -min. intervals. The number of peaks in the trace was counted for the first $2\frac{1}{2}$ -min. interval of each 5-min. interval, the results multiplied by 2, and the products averaged over an hour. The resulting number, divided into 300, gives the average period τ , in seconds, of a gust-and-lull considered as a unit. Furthermore, if this quantity is multiplied by the velocity of the wind in m. sec⁻¹, we obtain $2d$, twice the diameter of the eddies in metres. The necessity for the factor 2 will be made clear by a reference to Fig. 2, which must be understood as entirely diagrammatic. The actual structure of the wind is full of irregularities, well illustrated in the diagrams given by Sherlock and Stout (10).

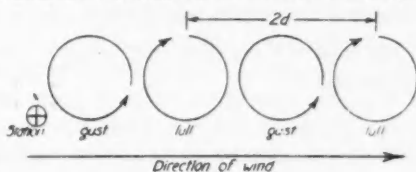


FIG. 2. Schematic diagram of gusts and lulls.

The results of these measurements are shown graphically in Fig. 3, in which the abscissas are wind velocities in miles hr^{-1} . As might be expected

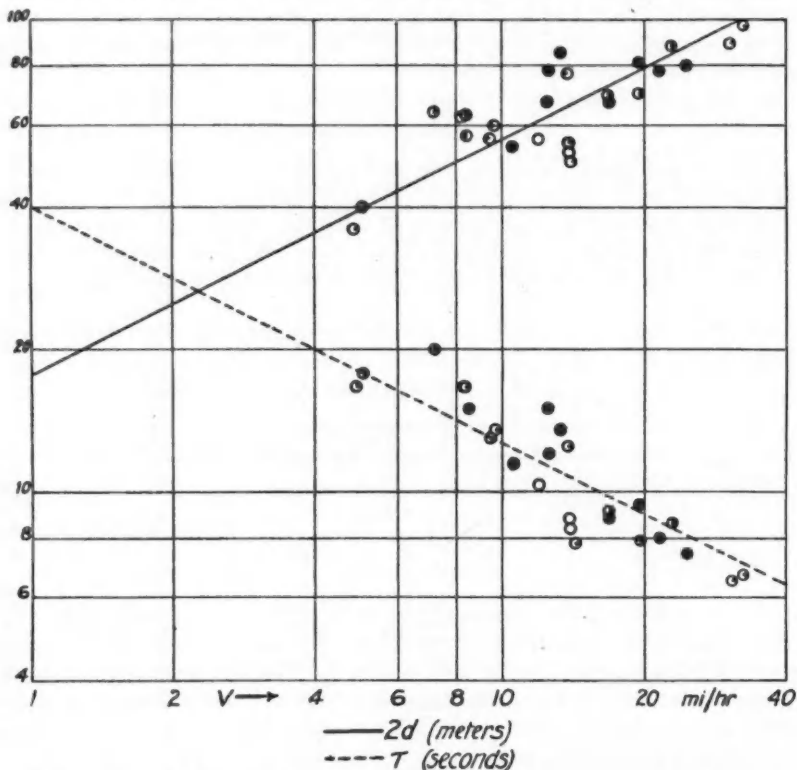


FIG. 3. Properties of gusts. Amount of black in small circles indicates amount of cloud.

from the nature of the data, the points show a good deal of scatter. However, the lines

$$\begin{aligned} \tau &= 40 V^{-0.5} \\ 2d &= 17.6 V^{0.5} \end{aligned}$$

represent the results fairly well.

We have now to inquire, what is the bearing of this on the problem of "galloping"?

The period of the vibrations known as "galloping" has been estimated at about 3 sec.; the excellent motion pictures of the phenomenon in the possession of the Hydro-electric Power Commission of Ontario, taken at Ellicott Creek near Buffalo during the glaze storm of Jan. 1, 1932, indicate that it is of this order. Now if we extrapolate the τ vs. V curve to $\tau = 3$ we get $V = 180$ miles hr^{-1} . No gusts with a period as low as 3 sec. or even 5 sec.

have been observed in this study. It is unlikely, therefore, that we are dealing with a resonance phenomenon; extremely unlikely, moreover, when we remember the completely irregular nature of the small variations in wind velocity. A similar irregularity manifests itself in the variations of the direction of the wind, and it has not been thought useful to attempt to discuss these quantitatively.

It may be objected that none of the records on which these figures are based have been obtained in glaze storms, an omission which will indeed be remedied at the first opportunity. However, a critical examination of the normal-speed records obtained under such conditions, as well as general meteorological considerations, suggest that especially small eddies in glaze storms are unlikely. In Fig. 3 the amount of cloud prevailing at the time is indicated by the amount of black in the small circles. It is evident that no difference in the effect of an overcast sky and of a clear sky is indicated by these results.

Meteorological Discussion of a Case of "Galloping"

The observations in the two preceding sections by no means close the question, and it is obviously necessary to adduce all possible evidence, and to test out all possible hypotheses, if the problem is to be finally solved. In pursuance of this idea it is considered desirable to set down here a discussion of the only reported case of "galloping" which has occurred sufficiently close to the instruments at St. Hubert to render such a discussion profitable.

Mr. J. W. Dunfield of the Southern Canada Power Company reported* a long-continued case of "galloping" on March 8, 1932, on the line between Farnham and St. John's, P.Q., which put the line out of service. This is a 48-kv. three-phase single circuit installation on two-pole H-frame wood construction, with suspension insulators. The direction of the line where "galloping" was observed is from east to west. The conductors are No. 00 stranded copper, span 400 ft., sag 7 ft., tension about 2000 lb., and spaced 7 ft. apart in a horizontal row. This line is only about 20 miles from St. Hubert. On this occasion heavy drifting snow was reported at St. Hubert, but not glaze. The temperature was 23.5° F. at 08h, having dropped fairly steadily from 32.5° F. at 20h the previous evening. It is thus rather likely that glaze had formed on the conductors during the night. The "galloping" was first observed at 08h, and "continued for several hours, and seemed to exist all along the line".

Fortunately a quick-run record of wind velocity is available for the period 10h to 12h on this date. Fig. 4 shows both the normal and quick-run records for March 7 and 8, and also a quick-run record for May 17, 1932, 10h to 12h, the latter being a normal wind of the same velocity as the one on March 8, and being inserted for comparison.

The reader will immediately notice in the quick-run records a difference in the *form* of the fluctuations. Those of March 7 and May 17 are usual, but those of March 8 are of a form which has not before been observed in these records. It is difficult to describe the forms of these curves, but

* Private communication to Mr. A. E. Davison.

attention may be drawn to the large and sudden increases and decreases in velocity on the 8th, often followed by comparatively steady velocity for 30 sec. or more. Before discussing these fluctuations we shall describe the general weather situation on this date.

Fig. 6 is a weather map for 08h on March 8, covering the eastern portion of the North American continent. The position of St. Hubert is indicated by two small concentric circles.

The controlling factor in the weather of the continent at this time was the great outbreak of polar air designated by P_e , which had pushed southwards and eastwards for five days over the portion of the continent east of the Rockies. In addition, a depression originating on March 5 as a wave in a front over the Gulf of Mexico had developed rapidly (28.6 in. at Wilmington, N.C., at 08h on March 6) and passed up the Atlantic coast. On March 7 the centre was off Boston, and by the morning of March 8 was over Cape Breton Island.

The air masses connected with this depression were, besides the great polar outbreak behind it, a notable invasion of tropical air over the Atlantic, and a return movement of old continental polar air over Quebec. On the morning of March 7 a somewhat diffuse cold front* had developed between this latter air mass and the main body of polar air, the difference of temperature across this front being about 10° F.

The southern portion of this front moved slowly eastwards, causing rain and snow in Michigan, Ohio, New York, and Quebec, and passed over St. Hubert at about 22h on March 7. It will be noted (see the top diagram of Fig. 4) that at this hour the wind completed a gradual change from N to SW. The thermogram, which is not reproduced, shows a sharp fall in the temperature at the same hour.

It was about eight hours after the passage of this front that the "galloping" was reported. The normal-run anemogram shows that at this time (08h March 8) the wind was 33 miles hr^{-1} from WSW, gustiness 0.7. At 10h the quick-run record was started, revealing the peculiar type of gustiness.

Two questions arise which must be settled as cases of "galloping", accompanied by appropriate meteorological records, become available. First, will winds of this sort cause "galloping", with or without ice on the cables; and second, are winds with this peculiar structure to be expected behind a front of the nature of the one which has been discussed? An anemometer with a very open time scale and small lag, such as has been described by Sherlock and Stout (9), would be of great value in this connection, especially if it could be set in motion immediately "galloping" is observed to occur.

The above case tends to support Niven's contention (8) that some peculiarity in the wind is responsible for the phenomenon. Many more cases must be studied, however, with sufficient observations available, before the real cause of "galloping" is determined.

* Readers unacquainted with the theory of fronts may find an excellent summary, written expressly for non-specialists, in Bulletin 79 of the National Academy of Sciences, National Research Council, Washington, 1931. (*Physics of the Earth—III. Meteorology.*)

PLATE I

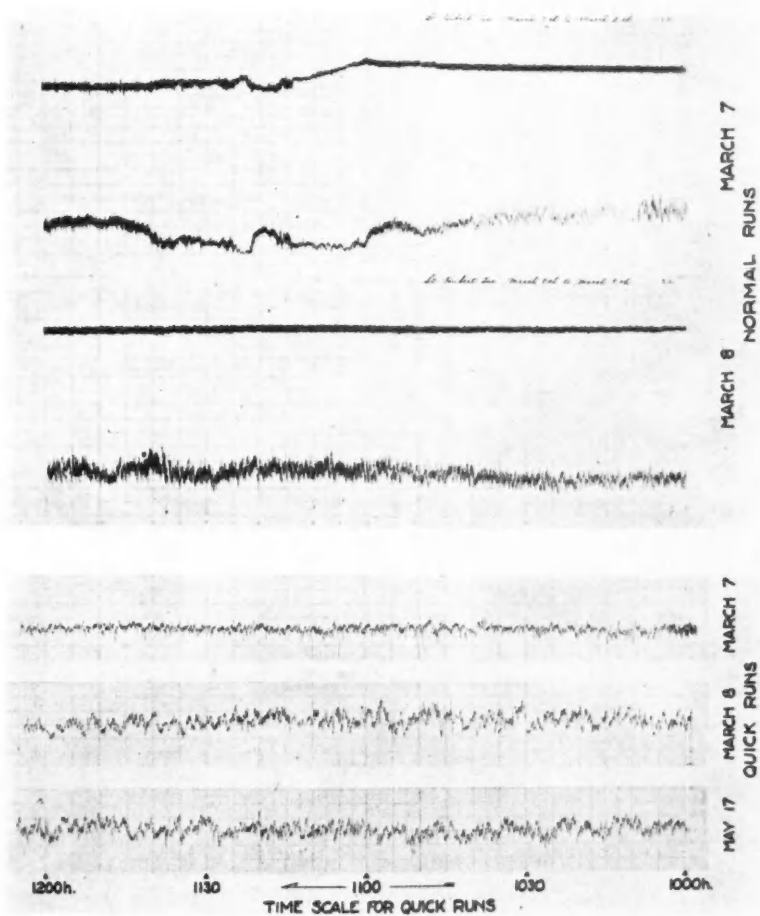
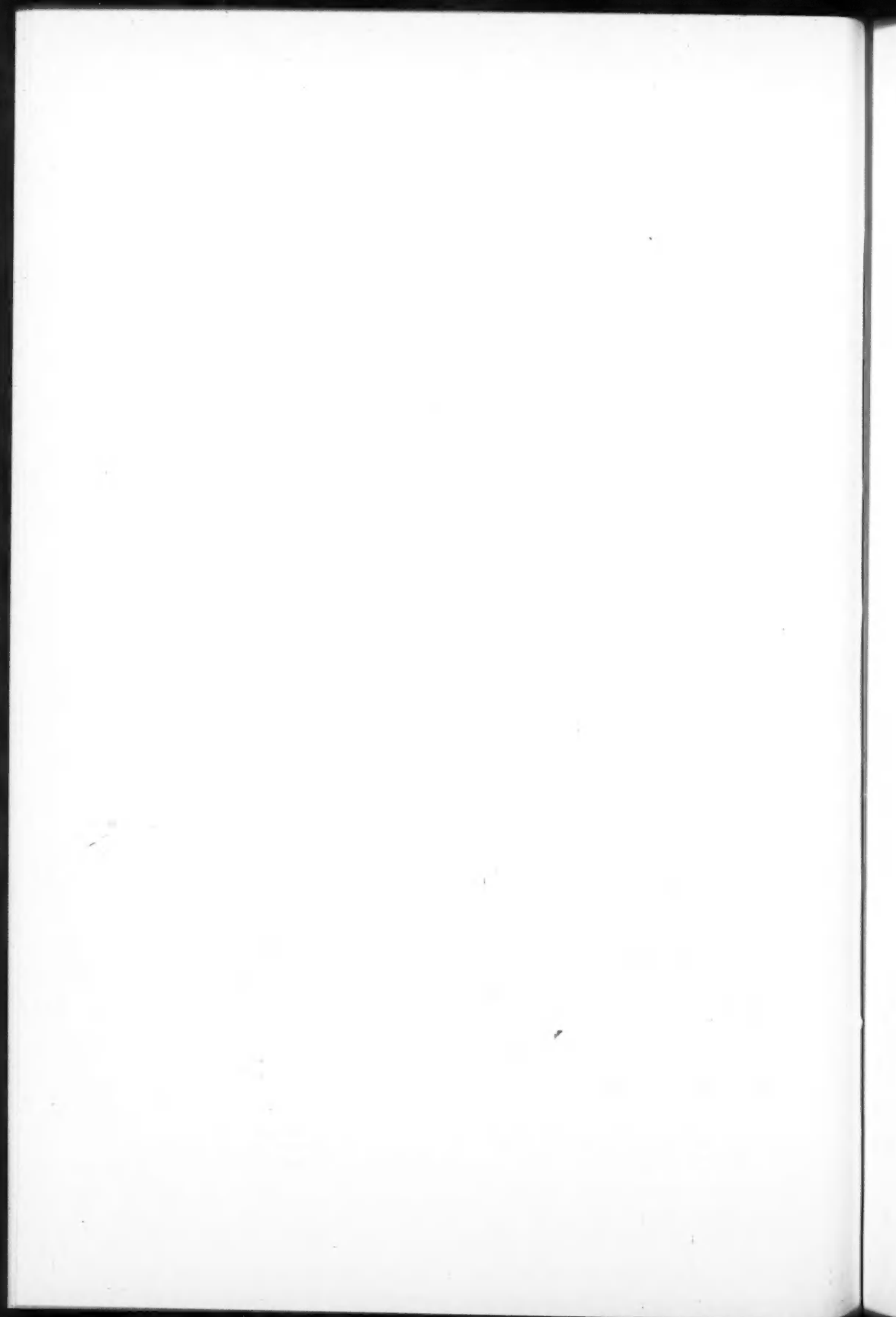


FIG. 4. Records of wind-structure.



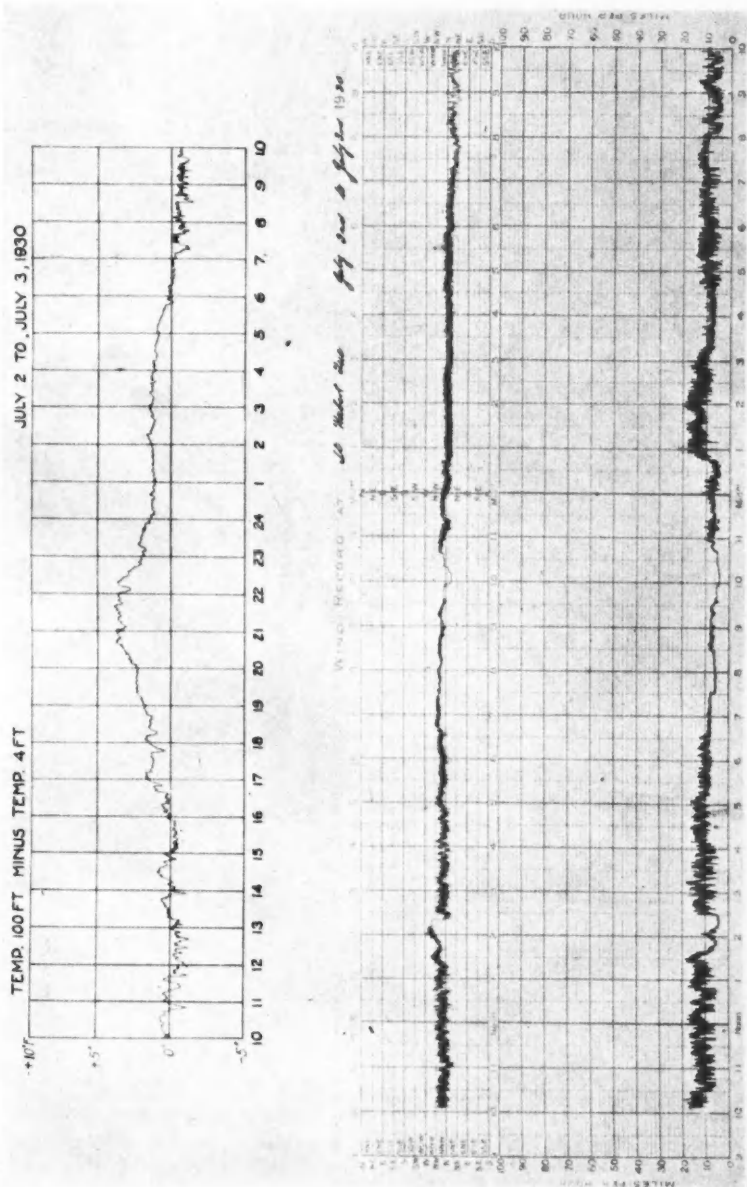


FIG. 5. Wind and temperature-gradient comparison.

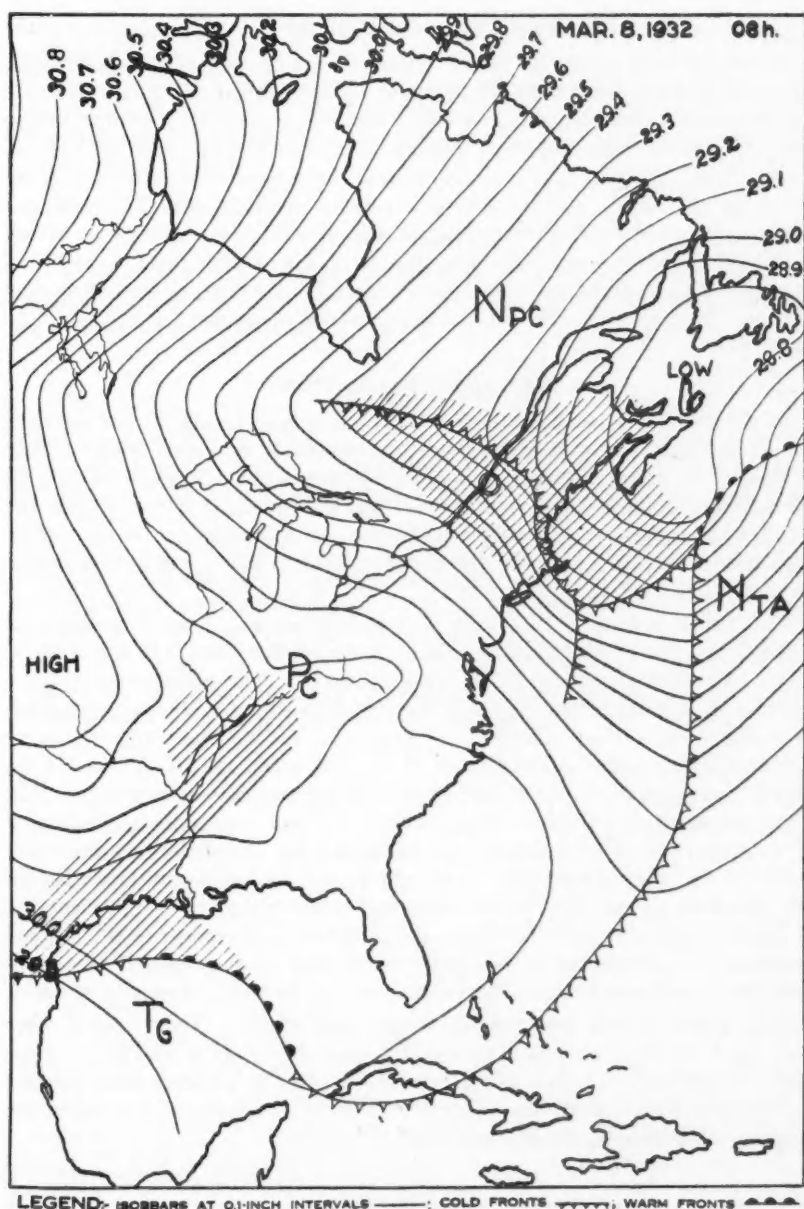


FIG. 6. The hatched area denotes precipitation falling at 08h 75th meridian time.
 The winds blow clockwise round the highs and counter-clockwise round the lows, following the isobars at about 500 m. above the surface, but tending into the lows and out of the highs at the surface. P_c, Continental (Canadian) polar air. N_{pc}, Returning continental polar air. T_g, Tropical gulf air. N_{TA}, Returning tropical Atlantic air.

Possible Effect of Ice on the Cable

The writer is indebted to Mr. J. Patterson for the suggestion that it may be possible that the fundamental cause of such large vibrations lies in a certain relation between the shape and size (in cross section) of the coating of ice on the conductors and the structure, velocity and direction of the wind at the time. DenHartog (4) and Davison (2) have shown that certain possible cross sections of ice, approximating to aerofoil sections, may give sufficient lift to account at least qualitatively for the upward movement of the wires. It would therefore seem desirable that if at all possible from a technical standpoint, measurements should be made of the ice coating when galloping occurs. In cases when the line has to be cut out of service this might not be impossible.

Unusually Steady Winds

Rapid vibrations of small amplitude, which lead to breaks in cable strands (3, 7), are due to eddies which form behind a cable (and also behind strings of insulators, tower members, etc.), in a steady wind. A given cable may vibrate in a number of configurations, but the essential factor is that the wind shall remain steady long enough for the vibration to be set up by resonance. In this connection the records shown in Fig. 5 will be of interest, as they are rather typical.

The lower portion of the figure shows the normal-speed direction and velocity records of the wind at St. Hubert from 10h July 2 to 10h July 3, 1930. The upper portion shows the difference in temperature between the top of a radio mast 100 ft. high and a screen at its base 4 ft. above the ground, over the same period, obtained by means of an automatically recording differential resistance thermometer. It will be noted that at about 14h on July 2, and again from 19h to 2245h, the wind became unusually steady. The upper record shows that at these times there was a considerable inversion of the lapse rate of temperature, which means that at these times the lower layers of the atmosphere were extremely stable, a condition unfavorable for the formation of the short-period gusts and absolutely prohibiting (according to Durst's theory referred to above) the presence of the longer ones. A wind as steady as is indicated at 20h might easily lead to short-period vibrations in a line. Such conditions often occur over flat country, especially at night.

Such steady winds are invariably very light winds. Niven tested wires in a wind tunnel with velocities ranging from 20 to 60 m.p.h. (8). As no natural wind of this speed could possibly be steady, at least over land, it would have been interesting to try velocities of the order of five miles per hour, to approximate actual conditions.

Conclusion

The writer would be glad to investigate, as far as available records allow, the meteorological conditions accompanying observed cases of "galloping" and of excessive short-period vibration. Only in this way can a proper

meteorological approach to the problem be made. The phenomenon of "galloping" especially is so infrequently observed that it will probably take many years before a satisfactory statistical treatment can be applied to the meteorological conditions which accompany it. The whole problem is obviously one which calls for the co-operation of the engineer, the physicist, and the meteorologist.

Acknowledgment

It is desired to acknowledge the kindness of Mr. A. E. Davison of the Hydro-electric Power Commission of Ontario in placing at the disposal of the writer a large file relating to the subject.

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NOTE ON SOLID-LIQUID EQUILIBRIA IN SOME TWO-COMPONENT SYSTEMS INVOLVING HYDROGEN CYANIDE¹

BY A. L. PEIKER² AND C. C. COFFIN³

Abstract

The freezing-point curves of the two-component systems hydrogen cyanide-water, hydrogen cyanide-formic acid, hydrogen cyanide-formamide and hydrogen cyanide-benzaldehyde have been determined. These systems are all of the simple eutectic type and show no evidence of molecular compound formation. A chemical reaction prohibited the investigation of solid-liquid equilibria in the case of halogen hydride-hydrogen cyanide systems.

Introduction

Incidental to an investigation of the effect of molecular compound formation on the velocity of certain reactions involving hydrogen cyanide, an attempt was made to determine the freezing-point curves of mixtures of hydrogen cyanide with water, hydrogen chloride, hydrogen bromide, benzaldehyde, formic acid and formamide. A chemical reaction in the liquid state at low temperatures was found to prohibit the study of solid-liquid equilibria in the halogen hydride-hydrogen cyanide mixtures. The data obtained for the other systems are here presented.

When this work was undertaken the only data on the freezing points of hydrogen cyanide-water systems were those of Gautier (3) and Lespieau (4). The work of the former was carried out before the formulation of the phase rule; the temperatures observed were not those of phase equilibrium and hence were valueless for the purpose in hand. The latter was concerned with concentrations of water in hydrogen cyanide up to only about one mole per cent. Quite recently however Coates and Hartshorne (1) published an account of a complete and apparently very careful study of the system, so that this part of the present paper would be superfluous were it not for the fact that the close agreement between the results of the two very different experimental procedures affords excellent confirmation of the essential reliability of each. While the method to be described is not capable of such accuracy as the heating- and cooling-curve method of Coates and Hartshorne, it nevertheless possesses certain advantages of speed and convenience in manipulation and has proved to be very suitable for handling such a toxic substance as hydrogen cyanide.

¹ Manuscript received January 9, 1933.

The work described in this paper was carried out during the Spring of 1929 under the direction of Prof. O. Maass in the Physical Chemistry Laboratory, McGill University, Montreal, Canada.

² Instructor in Chemistry, Trinity College, Hartford, Conn.; at the time graduate assistant in Chemistry, McGill University.

³ Associate Professor of Chemistry, Dalhousie University, Halifax, Nova Scotia, Canada; at the time holder of a fellowship under the National Research Council of Canada.

Experimental

The hydrogen cyanide, prepared as described in "Organic Syntheses", was passed through four calcium chloride tubes immersed in a water bath at about 40° C. and then into the apparatus of Fig. 1 at *N*. It was condensed in the bulb *K* which was surrounded with ether and solid carbon dioxide. When a quantity sufficient for the work in hand had collected in *K*, the generator was shut down and vented and the system to the right of the gas burette *B* was evacuated through *L* by a Hyvac pump on a balcony outside the building. (All traps, manometers, etc., e.g., at *O* in Fig. 1, were glass-sealed to a common vent line leading to the outside atmosphere.) The hydrogen cyanide was dried by distilling it back and forth several times through the phosphorus pentoxide tube connecting bulbs *K* and *H*. The middle third was then distilled to the storage bulb *G* and the tap *J* was closed. The vapor pressure measured on the manometer *M* was 27.0 cm. at 0° C. In Landolt-Bornstein Tabellen the value given is 26.8 cm. No noticeable

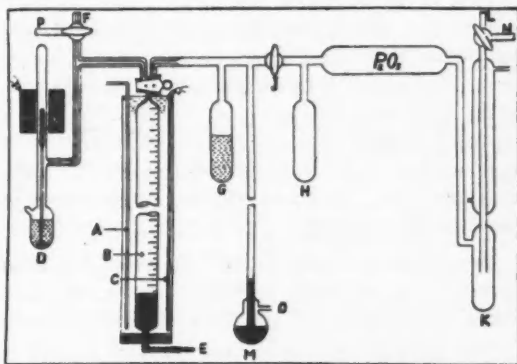


FIG. 1. Diagram of apparatus.

change in the vapor pressure or color of the liquid had occurred after it had remained three months in *G* at room temperature and about one-third of the original quantity had been removed for experiments. It is thus evident that no appreciable polymerization had occurred in the dried and purified liquid. The tubing of the generator system and of all the apparatus to the right of the phosphorus pentoxide tube was covered in a few days with the brown polymer of hydrogen cyanide.

The method of making up mixtures of known composition and determining their melting points was as follows. A weighed amount of distilled water was transferred from a pycnometer to the thin-walled bulb *D* (capacity about 5 cc.) through the side arm which was at once sealed off. The water was then frozen and kept at -78° C. while the system was evacuated through *F*. Three fusions and crystallizations *in vacuo* were usually required to remove all the dissolved air. By manipulating the stopcock and the levelling bulb (attached at *E*) of the gas burette, *B*, a known amount of hydrogen cyanide was transferred from *G* to *D* in a manner that is evident from the diagram. In order to keep the hydrogen cyanide in the gaseous state at a known temperature while its volume was being measured, the gas burette was surrounded with a large water jacket provided with an electric heater, *C*, and a tube, *A*, for air stirring. The temperature of the water was usually kept at about 35° C.

When the hydrogen cyanide had been added and the burette tap closed, the magnetic stirrer in *D* was started and the bulb was immersed in a large transparent Dewar flask filled with an alcohol-ether mixture and stirred by a current of dry air. The bath was cooled by adding pieces of carbon dioxide snow or "dry ice" until the solution in *D* froze, stopping the stirrer. The bath was then allowed to warm fairly rapidly until the contents of the bulb had melted sufficiently to allow the stirrer to function. At this point the bath was again brought under control and the rate of temperature rise regulated to about 0.1°C. in two or three minutes. This could easily be effected after some practice by adding powdered "dry ice" or increasing the stream of air through the bath. The thermometer in the bath and the vigorously stirred contents of *D* were kept under continual observation—temperatures and estimates of the amount melted being noted from time to time. When the crystals had almost disappeared the bath was slowly cooled again until the quantity of crystals had definitely increased. This was repeated several times for each mixture and the mean of the upper and lower temperature limits (usually differing by less than 0.2°C.) was taken as the melting point. Another addition of hydrogen cyanide was then made and the process repeated.

Temperatures below -30°C. were measured on a platinum resistance thermometer and above -30°C. on a mercury thermometer graduated in 0.2°C. and calibrated against a Reichenstalt standard.

The greatest source of error in such a method probably lies in the fact that the temperature of the bulb must lag behind that of the bath. However by allowing the bath temperature to change very slowly and at as nearly as possible the same rate during heating and cooling, as well as by using thin glass bulbs and small quantities of material, it is to be expected that such errors may be practically eliminated.

By connecting *D* to the rest of the apparatus with short lengths of capillary tubing, the volume of the vapor phase was kept small and any errors arising from this source were negligible.

The overlapping portions of the curves obtained in different runs always coincided, so that although the composition errors are necessarily cumulative during a run they are evidently quite small.

Results

The System Hydrogen Cyanide—Water

The results obtained for the hydrogen cyanide-water mixtures are plotted as circles in Fig. 2. It will be noted that within the probable limit of error the majority of the points fall upon the smooth curve which is drawn through the data of Coates and Hartshorne.

As these authors point out, the system is of the simple eutectic type and shows a marked inflection at about 35 mole per cent of hydrogen cyanide. On the water side of the diagram the curve is linear up to about 9 mole per

cent of hydrogen cyanide, which is unassociated at these concentrations. After this point, however, the apparent degree of association increases with increasing hydrogen cyanide content until at 35 mole per cent and -16.0°C . a close approach to complete association or non-solubility (*i.e.*, separation of another liquid phase) is indicated. By supercooling, Coates and Hartshorne actually found such a metastable two-liquid phase equilibrium for which they give the critical solution temperature as -24.0°C . and the critical composition as 35 mole per cent of hydrogen cyanide.

The slope of the curve on the hydrogen cyanide side shows that in dilute (less than about one mole per cent) solutions water is unassociated in hydrogen cyanide. The apparent degree of association increases continuously as the eutectic is approached.

The change in the appearance of the crystals with increasing hydrogen cyanide content may be roughly summarized as follows. From 0 to 20% of hydrogen cyanide—plate-like crystals (ice); from 50 to 60%—very small crystals (ice); from 60 to 80%—mixture of small crystals (ice) and long needles (hydrogen cyanide); from 80 to 100%—long needles (hydrogen cyanide). For some distance on either side of the eutectic the melting points of each crystal form could be noted. The two metastable solids occasionally observed by Coates and Hartshorne escaped notice.

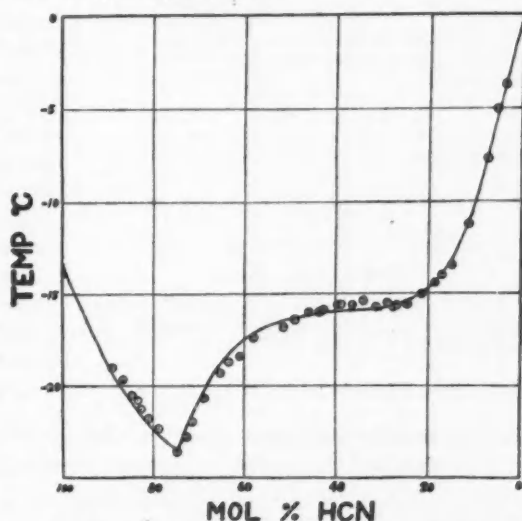


FIG. 2. Melting points of hydrogen cyanide-water mixtures.

The Systems Halogen Hydride—Hydrogen Cyanide

An attempt was made in the case of the halogen hydride—hydrogen cyanide mixtures to utilize the method described above with the modification that known amounts of pure dry hydrogen chloride or hydrogen bromide were condensed in *D* through the tube *P* by means of liquid air. It was found however that a chemical reaction which gave a white sandy precipitate took place in the liquid state at a velocity sufficient to render such a method impossible. In the hope that this reaction could be inhibited by keeping the mixtures frozen until the melting point could be quickly determined, recourse was had to a "bulb" method (2). Known amounts of the gases were condensed by means of liquid air in small glass bulbs which were then sealed

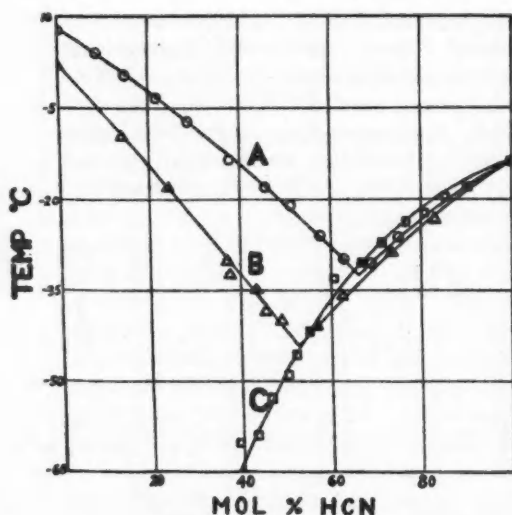


FIG. 3. Melting points of mixtures of hydrogen cyanide and (A) formic acid, (B) formamide, (C) benzaldehyde.

gave them the formulas $\text{HCl} \cdot \text{HCN}$ and $3\text{HBr} \cdot 2\text{HCN}$.

The Systems Benzaldehyde-Hydrogen Cyanide, Formic Acid-Hydrogen Cyanide and Formamide-Hydrogen Cyanide

The experimental method in these cases was similar to that used for the water-hydrogen cyanide system. The results are plotted in Fig. 3. As is apparent from the curves each of the three systems is of the simple eutectic type showing no indication of molecular compound formation. Indeed the opposite tendency is evident—self-association characterizing every branch of the curves. On the hydrogen cyanide side of the diagram the molecular weights calculated from the slopes of the first half of the curves show that each solute exists as approximately double molecules. The slopes of the branches on the other side of the diagram indicate a considerable and fairly constant association.

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off and kept at -190°C . until all was in readiness for the melting point determination. This method was unsuccessful, however, as the reaction seemed to take place to a considerable extent during the preliminary melting necessary to mix the contents of the bulbs. This was particularly true of the hydrogen bromide mixtures which reacted violently and several times exploded as they melted. The solid reaction product, while always white in the case of hydrogen chloride, varied from a light pink to a deep red color in the hydrogen bromide reactions. These compounds were presumably those prepared and analyzed by Gautier (3) who

THE SULPHUR CONTENT OF CRUDE NAPHTHA FROM TURNER VALLEY IN RELATION TO REFINING PRACTICE¹

By J. W. SHIPLEY²

Abstract

Typical samples of Turner Valley naphtha were examined for their sulphur content and for their behavior towards common refining treatments. The sulphur content ranged from 0.15 to 0.19% and consisted of sulphides, organic disulphides and mercaptans. Thiophenes were present, but in very small amounts. No free sulphur or peroxides were detected. Distillation concentrated the color and color producing compounds and the gum and gum producing compounds in a small high-boiling residual fraction. Exposure to light, even in the absence of air, led to the development of a yellow color and to gum formation in all undistilled samples, treated or untreated. Distillation up to 165° C. yielded a fraction (95% by volume) which remained colorless and free from gum formation when exposed to the air and to light for over eight months.

Recovery of the propane and butane is recommended, and the treatment of the crude naphthas with sodium hydroxide solution or with sodium hydroxide and sodium sulphide solutions followed by distillation is suggested as a means of producing a fairly satisfactory gasoline. The caustic wash alone reduces the sulphur content to about 0.1%, while the alkaline-sodium sulphide wash reduces it to about 0.05%.

Sulphuric acid, sodium hypochlorite, caustic wash, sodium plumbite and cupric chloride treatments were examined and the refined products compared for sulphur content, sweetness, corrosion, gum formation and color. None of these treatments prevented gum formation or the development of color when the treated samples were exposed to light.

Introduction

Crude naphtha is separated from the Turner Valley (45 miles southwest of Calgary, Alberta) wet gases by permitting the casinghead gas to expand from the well pressures to a pressure of 200–250 lb. in baffled metal separators. The drop in pressure, with consequent expansion and cooling, causes the separation of some of the more condensable hydrocarbons in liquid form. This liquid coming from the separators forms the principal source of motor fuel from the Turner Valley wells. The yield of liquid naphtha varies from well to well, the highest being about one barrel per 20,000 cu. ft. and the lowest, one barrel per 1,000,000 cu. ft. of gas. The residual gas is either passed through a scrubbing plant and piped to Calgary, or is permitted to burn in open flares in the valley. Since the production of gas from the wells is many times that of the local requirements, most of the waste gas up to a few months ago was burned in the open flares. The reflection of light at night above the valley could be seen for miles, and advertised far and near the magnitude of the wastage of fuel connected with the recovery of a small fraction of liquid fuel from the major resource of natural gas arising from the wells of the valley. Restrictive measures on the part of a conservation committee of the provincial government are designed to bring about a larger recovery of liquid hydrocarbons from the wet gas, and to conserve the gas resources of the valley.

¹ Manuscript received October 11, 1932.

Contribution from the Chemistry Department, University of Alberta, Edmonton, Albert Canada.

² Professor of Chemistry, University of Alberta.

The crude naphtha from the separators contains highly volatile hydrocarbons and sulphur compounds which make it desirable to refine the naphtha before using it as a motor fuel. At present the general practice in the valley is to "weather" the crude naphtha in tanks open to the air, or even to heat the tank contents with steam in order to drive off the more volatile hydrocarbons, principally propane and butanes. This involves a loss of up to 30% of liquid hydrocarbons, the average loss in 1930 being probably in the neighborhood of 12% of the liquid naphtha recovered in the separators. No recovery of these valuable hydrocarbons has as yet been attempted, although elsewhere on the continent both butanes and propanes are recovered and sold as fuel or for lighting purposes. The process of removing these volatile hydrocarbons is known as "stabilizing" the naphtha.

After "stabilizing", the naphtha is sent to the refinery where it is either mixed with crude oil from other sources and refined, or is treated for the removing or changing of the objectionable sulphur compounds. It is then marketed. The latter treatment is the practice in the smaller refineries, and recently processes have been adopted which provide a fairly satisfactory motor fuel without admixture of gasolines derived from any other source than the Turner Valley naphthas. A certain amount of the naphtha is disposed of as a motor fuel without being refined. If such a fuel comes directly from the separator the volatile hydrocarbons are largely lost before they enter the combustion chamber of the engine. This objection cannot be raised with regard to the naphtha from the stabilizers but, whether stabilized or not, crude naphthas contain the objectionable sulphur compounds. Whether these sulphur compounds give rise to corrosion or not is a matter of opinion rather than of demonstration, but the odor of hydrogen sulphide and mercaptans makes them undesirable constituents of automotive fuels.

Several analyses of crude naphtha from the wells of Turner Valley have been made by Mr. R. Rosewarne, engineer of the Fuels and Fuel Testing Division of the Department of Mines, Ottawa. These show a propane content varying from 2 to 10.8%; butanes, 8.8 to 20.3%; pentanes and higher hydrocarbons, 61.4 to 89.2%. Methane and ethane, up to 5% of each, are also constituents of some of the crude naphthas. The specific gravity ranged from 0.62 to 0.70. No data respecting the sulphur compounds, excepting the total sulphur content of the crude naphthas, were obtainable. The total sulphur content of the crude naphthas was, however, known to range between 0.10 and 0.20%.

Corrosion of telephone lines and telephone apparatus adjacent to the Turner Valley field has been reported from time to time, the corrosion being attributable to the sulphur compounds emitted into the air from leaking gas pipes, blowing of wells, the gases from stabilizer tanks and the oxidation products from the open flares in the valley. Corrosion of exposed metal parts in the operating plants in the valley was also observed but, considering the exposure to which these were subjected, the damage was relatively small. This relative freedom from corrosion is probably due to the lack of

moisture in the air and not to the absence of sulphur compounds. Tanks and pipes covered with aluminium paint appeared to be well protected against corrosive attack. No evidence could be obtained from the condition of the vegetation in Turner Valley that there was a sufficient concentration of sulphur dioxide in the atmosphere to adversely affect the growth of plants. At all times and places the vegetation appeared normal.

The investigation described in this paper was carried out for the purpose of learning something of the nature of the sulphur content of the Turner Valley naphthas, particularly in relation to refinery practice. The refining processes are largely concerned with either removing the sulphur compounds from the naphtha, or rendering them non-objectionable from the standpoint of odor and corrosion, and in securing a product free from color and the tendency to gum formation in storage.

Material

Through the courtesy of Mr. W. Calder, Chief of the Petroleum and Natural Gas Division of the Provincial Department of Lands and Mines, and Mr. C. W. Dingman, District Petroleum and Gas Engineer of the same department, samples were secured from the separators attached to four representative wells, *viz.*, McLeod No. 4, Sterling Pacific No. 1, Spooner No. 1 and East Crest Nos. 1 and 2A combined. The samples were taken direct from the bottom of the gauge glass of the separators and kept in brown glass bottles previously well scrubbed with the gas from the separators, and at no time exposed to the air or to the light. Small portions for analysis were removed from the containers by making use of the pressure generated by warming the bottles slightly.

Experimental

Since the investigation was not concerned with distilling practice the separate samples were not examined for volatility, but a 100-cc. sample made up of equal parts of the four samples was distilled according to standard practice. The first drop appeared at 30° C.; 10% came over between 30° and 50°; 15% between 50° and 55°; 10% between 55° and 70°; 10% between 70° and 80°; 10% between 80° and 90°; 10% between 90° and 100°; 20% between 100° and 120°; and 10% between 120° and 165° C. The liquid residue (5%) was yellowish in color but no solid separated on cooling. The distillates were colorless but on standing for several weeks exposed to the light a slight yellow color developed in the last fraction. The specific gravity of the mixed naphthas before distilling was 0.687 at 20° C.

Sulphur Content

All four samples reacted strongly with doctor solution (sodium plumbite) forming, without the addition of sulphur, yellow to black precipitates, and the naphthas on filtering were yellow. On the addition of sulphur all precipitates became brown or black, excepting the East Crest sample which

changed from yellow to brown. The presence of hydrogen sulphide and disulphides in all of the samples modified the characteristic color reactions of lead plumbite on the mercaptans.

All four naphthas contained alkyl disulphides and mercaptans and all gave positive reactions to the indophenene test, indicating the presence of thiophenes.

An approximate analysis to determine the various kinds of sulphur compounds present in the naphthas was carried out in accordance with a method suggested by Youtz and Perkins (7). The total sulphur was determined by the standard combustion method using a burner and absorption pipette. The sample (25 cc.) was then shaken for 20 min. with an equal volume of a saturated aqueous solution of sodium carbonate to remove the hydrogen sulphide, and the sulphur again determined in the naphtha by combustion. The disulphides were then removed by shaking the naphtha for 20 min. with an equal volume of a saturated aqueous solution of sodium sulphide, and the residual sulphur determined in the naphtha by combustion. Finally, the naphtha was shaken with an equal volume of a saturated aqueous solution of mercuric chloride to remove mercaptans, the residual sulphur in the naphtha again being determined as above. After each treatment the naphtha was washed twice with water before the sulphur was determined. The results according to this approximate separation are shown in Table I.

TABLE I
AMOUNTS OF VARIOUS KINDS OF SULPHUR COMPOUNDS PRESENT IN
TURNER VALLEY NAPHTHAS

Sample	% S Total S	% S as H ₂ S	% S as di- sulphides	% S as mer- captans	% S undeter- mined
McLeod No. 4	0.162	0.057	0.044	0.032	0.029
Sterling Pacific No. 1	0.162	0.027	0.031	0.080	0.024
Spooner No. 1	0.148	0.050	0.053	0.022	0.023
East Crest Nos. 1 and 2A	0.186	0.055	0.041	0.068	0.022

After the removal of the sulphides and disulphides, all the naphthas gave the characteristic canary yellow coloration with sodium plumbite; they became orange-colored and yielded a precipitate on the addition of sulphur. This proved conclusively the presence of mercaptans. After shaking with mercuric chloride solution, no positive test for either mercaptans or thiophenes could be obtained. The thiophenes therefore are contained in the mercaptan sulphur, but all tests for thiophenes indicated their presence in but very small quantities.

Corrosion tests on the untreated naphthas, using the copper strip method, were all positive and the order of intensity in the blackening of the copper was, first, Sterling Pacific, followed by Spooner, McLeod and East Crest in the order named. Shaking with mercury produced a heavy black precipitate.

Treatment with Sulphuric Acid

Samples of the four gasolines were placed in wash bottles and treated with an equal volume of 96% sulphuric acid. Air was drawn through in such a manner as to mix the gasoline and acid. After 30 min. the samples were washed with a saturated aqueous solution of sodium carbonate and with water, and analyzed for sulphur. The sulphur content in the McLeod No. 4 and Spooner No. 1 samples was reduced to about 0.01% by this treatment, while in Sterling Pacific and East Crest it was reduced to 0.04%. All the treated naphthas were negative to doctor solution. The air drawn through the mixture of acid and naphtha was passed into a standard alkali solution and the volume of sulphur dioxide evolved determined. The amount of sulphur dioxide evolved was negligible. Apparently this treatment was very effective for the removal of sulphur when it was not combined as mercaptans. Both Sterling Pacific and East Crest were relatively high in mercaptans.

Samples of the four naphthas were mixed in equal proportions and a series of experiments carried out to find the minimum quantity of sulphuric acid required, with 20 min. shaking, to remove the sulphur compounds. A 25-cc. sample of the mixed naphthas was first shaken with an equal volume of 20% sodium hydroxide solution and washed with water. The minimum proportions by volume were found to be about 1 of 96% acid to 15 of the naphtha. A smaller proportion of concentrated acid, or dilute acid, required a longer period of contact before the naphtha was entirely sweetened. The sulphur content of the mixed naphthas after treatment was about 0.01%.

During these experiments it was found that only a very small loss, 2% by volume, of naphtha occurred if the temperature of the mixture was kept below 20° C. The reaction with sulphuric acid produced considerable heat and the sulphuric acid was blackened, but very little evolution of sulphur dioxide took place.

The naphthas sweetened by the sulphuric acid treatment and washed with sodium hydroxide solution and water were kept under observation for over a year. The water-white products gradually assumed a brownish yellow color and there was a small deposit of gum. The samples were exposed on the laboratory table. Acidity to methyl orange developed but the samples remained sweet to doctor solution. They developed however a highly corrosive action towards copper. This action could be prevented by washing with sodium hydroxide solution. No blue color was formed on shaking with potassium iodide-starch solution.

Treatment with Sodium Hypochlorite

A sodium hypochlorite solution was prepared by saturating in the cold a 20% solution of sodium hydroxide with chlorine and then making it alkaline by the addition of one-fifth of its volume of the 20% alkali solution. Neither the alkalinity nor the available chlorine was determined. Equal volumes (25 cc.) of the freshly prepared sodium hypochlorite solution and of the naphthas were shaken together in 100-cc. flasks for 20 min., separated, and then the naphthas were washed twice with water. Three of the naphthas

were not rendered completely sweet by the above treatment but longer contact with the sodium hypochlorite solution reduced the sulphur content and the positive test towards doctor solution. The single 20-min. treatment rendered Spooner No. 1 naphtha sweet and reduced the sulphur content to 0.05%, while in the other three samples it was reduced to about 0.10%. The sodium hypochlorite treatment preceded by a caustic wash with sodium hydroxide solution rendered all four of the naphthas sweet and reduced the sulphur content to about 0.05%. Possibly a more strongly alkaline solution of sodium hypochlorite or longer contact with the sodium hypochlorite solution as used would have rendered the naphthas sweet without a previous caustic wash.

The naphthas were rendered water-white by the sodium hypochlorite treatment but developed a yellow color on standing for a few hours. This color developed much more slowly when the samples were kept in the dark.

Naphthas treated with sodium hypochlorite, unless followed by a caustic wash, were found to give a heavy black precipitate with mercury. Treatment with chlorine gas was attempted but the experiments were not continued as there was a tendency for the naphthas to emulsify when washed with alkali or water.

Treatment with Cupric Chloride

Samples of the naphthas were given a preliminary wash with sodium hydroxide solution, followed by shaking for 20 min. with an equal volume of a saturated aqueous solution of cupric chloride. The treated naphthas, after washing with water, were sweet to doctor solution but the sulphur content was very little reduced. There was a tendency towards emulsifying on shaking with the cupric chloride solution. A previous wash with sodium hydroxide solution prevented the blackening of the cupric chloride solution due to the removal of the sulphides. After treatment the naphthas were filtered through "diotite", a diatomaceous earth, and set aside for observation. They slowly became yellow and gum formation was observed, but they remained sweet towards doctor solution for over a year.

Peroxides

Qualitative tests were made for peroxides, using titanous chloride, but no reaction could be detected. Samples of naphtha left exposed to light for six months in stoppered bottles containing air also failed to show any formation of peroxides. The four naphthas were also tested by shaking with potassium iodide solution to which a little starch was added. None gave a blue coloration. Apparently the naphthas do not contain any strongly oxidizing compounds.

Coloration

The samples of naphtha when collected, and after keeping for over a year in brown glass bottles, had very little color. McLeod No. 4 and Sterling Pacific had a slight yellowish tinge, while Spooner No. 1 was just faintly yellow, and East Crest was water-white. Samples of these naphthas were set aside for observation of the color changes under varying conditions.

Set No. 1 was kept in stoppered glass bottles, but no attempt was made to keep them air-free. They were exposed on the laboratory table at times to direct sunlight and strongly lighted at all times during the day. A yellow color developed in a few days, the depths of color being in the order mentioned above. After a few months, gum was observed to form on the sides and bottom of the flasks, and the amount appeared to be in the order of the depth of color. East Crest after a year's exposure was only faintly yellow and gum formation was not marked.

A second set of samples was placed in a dark cupboard. These developed color very slowly and no gum formation could be observed after a year's exposure.

A third set was wrapped in black paper and set aside in the cupboard. No color development or gum formation whatsoever could be observed.

A fourth set was exposed to the sunlight for a day and then placed in the dark. No further development of color occurred.

Samples of naphtha which were placed in bottles in which the air had been displaced by carbon dioxide became yellow and gum was formed, as was the case when the samples were in contact with air. Evidently the color development and gum formation is attributable to exposure to the light and not to the presence of air.

A mixed sample of four naphthas was distilled and various fractions set aside. The fractions distilling below 165° C. were colorless and remained so for eight months when exposed to the same light conditions as was set No. 1. No gum formation occurred in these fractions. Fractions above 165° C. were yellowish, the color deepening to brown as the distillation proceeded. The color of these fractions deepened under the light conditions mentioned above, gum formation occurred and a black deposit formed in the higher fractions.

Filtering the colored naphthas through "diotite" removed the yellow color, but on exposure to light the color again developed and gum formation took place.

Corrosion

The four naphthas were strongly corrosive to copper and gave heavy black deposits when shaken with mercury. A caustic wash followed by water removed the corrosive compounds or changed them so that the copper test was negative. This accounts for the fact that several of the refineries now treat Turner Valley naphthas with an alkali wash, and market the product without any other treatment. The sulphuric acid treatment followed by a caustic wash gave a naphtha at first non-corrosive to copper, but on standing for some time it became corrosive. Treatment of the naphtha with sodium plumbite, without the addition of sulphur, leaves the naphthas with a canary yellow color, and elemental sulphur must be added to change these yellow compounds to colorless. A slight excess of free sulphur over the amount necessary makes the naphtha decidedly corrosive. Consequently the doctor treatment must be very carefully regulated in regard to the addition of sulphur.

Treatment with free chlorine or with sodium hypochlorite and washing with alkali solution gave a non-corrosive product, providing the washing was thorough. It was found however that, on standing, a yellow color developed in the naphtha and the corrosion test became positive.

Treatment of the naphthas with sodium hydroxide solution followed by a saturated cupric chloride solution and washing gave a sweet non-corrosive product, remaining so for 12 months.

Filtering through "diotite" did not remove the corrosive substances in the naphthas nor were they rendered sweet to sodium plumbite.

Discussion

The four naphthas examined had a total sulphur content ranging from 0.148% to 0.186%, and consisting of varying amounts of sulphides, disulphides and mercaptans. A caustic soda wash removed the sulphides and probably some of the lower mercaptans, reducing the sulphur content to the neighborhood of 0.1% and giving a non-corrosive product which remained colorless for a time. A large part of the mercaptan content, however, remains in the naphtha as such and, on standing, a yellow color develops and gum formation occurs. Nevertheless this refining process, becoming more popular in the treating of Turner Valley naphthas, gives a gasoline much superior to the crude casinghead naphtha or the "stabilized" naphtha marketed in the valley. The practice of coloring the naphtha blue masks the yellow color that develops on standing. It is extremely interesting that the caustic wash should render the gasoline negative to the copper strip corrosion test, when only half or less than half of the total sulphur content has been removed. The advantage of the caustic wash over other methods lies in its simplicity and cheapness, making it possible to produce a salable product at a considerably lower price than if the naphtha were mixed with crude oil or gasolines from other sources and refined by more expensive processes. The disadvantage of alkali-washed Turner Valley naphthas lies in the fact that they still contain some of the objectionable odorous sulphur compounds (mercaptans) and too high a content of volatile hydrocarbons.

Turner Valley naphthas are susceptible to the sodium hypochlorite treatment following a caustic wash, which transforms the mercaptans to the less objectionable disulphides, removes the sulphides and reduces the sulphur content to 0.05% (3, 4, 5, 6). Naphthas so treated remained sweet but developed a yellow color in time, and gum formation followed. The cupric chloride treatment following a caustic wash rendered the naphthas sweet and the development of a yellow color and gum formation seemed to be retarded, but the sulphur content was not reduced to as low a proportion as with the sodium hypochlorite treatment. Nevertheless the naphthas treated with cupric chloride remained non-corrosive for over a year.

The alkaline plumbite (doctor solution) treatment accompanied by the addition of sulphur renders the naphthas sweet, but care must be exercised in the addition of the sulphur to avoid any excess, as even a slight excess was found to render the naphthas corrosive.

Treatment with sulphuric acid followed by a caustic wash produces water-white naphthas, sweet for an indefinite period and having a very low sulphur content. On standing, however, a yellow color slowly develops, accompanied by gum formation. Previous washing with sodium hydroxide solution would diminish the amount of sulphuric acid required to oxidize the sulphur compounds, but it would be necessary to find out whether such additional treatment would be cheaper in practice. No excessive loss of hydrocarbons follows the use of concentrated sulphuric acid, providing the temperature is kept down to 20° C.

None of the treatments carried out in this investigation produced a naphtha remaining colorless for an indefinite time (1, 4). Neither did any of the treatments render the naphtha free from the development of the objectionable gummy compounds. Exposure to light always resulted in the development of a yellow color and the precipitation of gum. On the other hand all crude and treated samples were free from color development and gum formation when protected from the light. It would seem desirable, therefore, whatever the method adopted in refining Turner Valley naphthas, that care should be exercised in protecting the refined product from exposure to light. The development of the yellow color (4) in itself is probably harmless but gum formation is certainly undesirable.

A sulphur content of 0.04% in a gasoline is non-corrosive in motor engines (2), while a content of 0.15% is appreciably corrosive. The Turner Valley naphthas, if untreated, are to be considered as corrosive, while, if subjected to a caustic wash reducing the sulphur content to 0.1%, they are in the threshold region of corrosion where the limits of tolerance have not as yet been established. No doubt refining practice which reduces the sulphur content to 0.05% gives a more desirable product so far as our present knowledge of the relation of sulphur content to corrosion goes, but it is a moot question whether the extra cost of refining required to reduce the sulphur content from 0.1% to 0.05% without removing the tendency to gum formation is justified. Refinery practice which renders the Turner Valley naphthas relatively odorless and reduces the sulphur content to about 0.1% ought to be satisfactory, providing the refined product is not later exposed to light. Coloring the naphtha will mask the yellow color developed on exposure to light but will not prevent the formation of gum. Distillation concentrates the color and gum producing properties in the high boiling fractions. If the separation of the propane and butanes was economically practicable, refining practice for Turner Valley naphthas would probably be confined to a caustic wash with or without sodium sulphide or polysulphides followed by distillation. The first distillates would contain the propane and butanes, from 5 to 30%; the middle fraction up to 165° C. from 65 to 90%, marketable gasoline, and a high boiling fraction of about 5% containing the colored and gum producing properties could be treated along with the crude oils produced in the valley. A comparison of methods for treating the crude oils of the valley has not as yet been made.

It is recognized that other factors, particularly economic ones, enter into the choice of a method of refining Turner Valley naphthas. It is also recognized that this investigation falls far short of determining the proper concentrations of reactants or the optimum conditions under which these reactants would be most effective in refining practice, but the results at least indicate what treatments are most likely to give to the consumer a refined gasoline which is satisfactory from the standpoints of sulphur content, corrosion, color and gum formation.

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STUDIES ON REACTIONS RELATING TO CARBOHYDRATES AND POLYSACCHARIDES

XLIV. SYNTHESIS OF ISOMERIC BICYCLIC ACETAL ETHERS¹

BY EARLAND G. HALLONQUIST² AND HAROLD HIBBERT³

Abstract

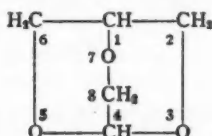
Two isomeric bicyclic acetal ethers, namely, 3, 5, 7-trioxabicyclo [2, 2, 2] octane* and 3, 6, 8-trioxabicyclo [3, 2, 1] octane, have been synthesized by the action of aqueous potassium hydroxide on 1,3-bromoethylidene glycerol 2-benzoate and 1,2-bromoethylidene glycerol 3-benzoate respectively. Both isomers are crystalline products possessing similar solubilities in organic solvents and high vapor pressures. They show no indication of interconvertibility on solution in solvents, or under the influence of heat. On hydrolysis they yield glycolic aldehyde and glycerol.

A new method for the synthesis of cyclic acetals from simple open-chain acetals is described.**

Introduction

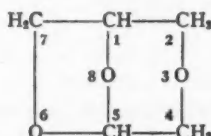
For a thorough understanding of the nature and properties of carbohydrates, and especially of polysaccharides, three factors call for especial consideration, namely: (1) the position in space of the hydroxyl groups; (2) the nature of the carbon-oxygen valence angle in the ring and its bearing on the stability of the latter; (3) the nature and tendency towards polymerization of carbon-oxygen ring compounds, and the stability and polar character of the resulting polymerized product.

Some ten years ago (8) preliminary experiments were carried out on the action of alkalis on bromoethylidene glycerol and a crystalline product isolated to which was tentatively assigned the structure of (A) or (B).



(A)

3, 5, 7-Trioxabicyclo [2, 2, 2] octane



(B)

3, 6, 8-Trioxabicyclo [3, 2, 1] octane

In the light of recent developments on the magnitude of the carbon-oxygen valence angle (1, 6) it seemed of interest again to take up the study of this subject with the object of securing additional information on the structure and properties of -C-O-C- ring linkages, and to investigate first of all bicyclic derivatives containing neither hydroxyl nor carbonyl groups.

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²Contribution from the Division of Industrial and Cellulose Chemistry, McGill University, Montreal, Canada, with financial assistance from the Canadian Pulp and Paper Association.

³Postgraduate student and holder of a fellowship under the National Research Council of Canada.

⁴Professor of Industrial and Cellulose Chemistry, McGill University.

* This system of nomenclature is in accordance with that suggested by the Division of Chemical Abstracts, American Chemical Society.

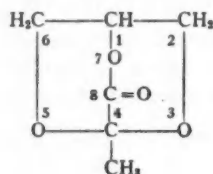
** This method is to be applied later to carbohydrates and related compounds.

It has been found that the action of aqueous potash on 1, 3-bromoethylidene glycerol 2-benzoate and 1, 2-bromoethylidene glycerol 3-benzoate yields two isomeric substances, namely, (A) and (B) respectively, the structure of which has been definitely settled by the procedure outlined in the Experimental Part.

The bicyclic derivative (A) is formed much more readily than (B) and this property can, in fact, be utilized for the isolation of 1, 2-bromoethylidene glycerol from a mixture of the bromoethylidene derivatives. Both isomers have a well-marked crystalline character, a high vapor pressure, and similar solubilities in organic solvents, while neither shows any tendency to pass over into the other in solution or under the influence of heat.

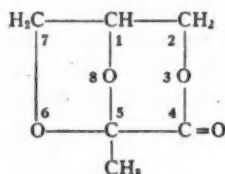
It is of interest that a structural model of (A) employing the same valence angle for $-C-O-C-$ as for $-C-C-C-$ linkages represents a highly symmetrical substance.

The fact that interconversion of the one into the other does not readily take place is probably to be associated with the absence of strongly polar groups, inasmuch as Haskelberg (4) has recently found that on heating together glycerol and pyruvic acid two isomeric bicyclic derivatives, namely, 4-methyl-3, 5, 7-trioxabicyclo [2, 2, 2] octan-8-one (C) and 5-methyl-3, 6, 8-trioxabicyclo [3, 2, 1] octan-4-one (D), are formed, one of which is a solid (C) and the other a liquid (D) and to which the author has arbitrarily assigned the following structures:



(C)

4-Methyl-3, 5, 7-trioxabicyclo
[2, 2, 2] octan-8-one



(D)

5-Methyl-3, 6, 8-trioxabicyclo
[3, 2, 1] octan-4-one

without, however, providing any experimental proof. The point of interest is the discovery that the liquid isomer (D) on being allowed to stand in the presence of hot water passes over into the isomer (C), which separates out in the crystalline state on cooling.

A study of this new type of compound is of interest from the point of view of the stability of heterocyclic carbon-oxygen ring compounds, the carbon-oxygen valence angle in heterocyclic rings, the properties of polysaccharides, and the Hibbert-Michael ring partition principle.

Synthesis of Cyclic from Simple Open-chain Acetals

Due to the difficulty in preparing various substituted halogen and hydroxy-cyclic acetals, it was found desirable to devise a new method for their synthesis. The process described consists in merely heating a mixture of the polyhydroxy

derivative (for example, glycerol) with an open-chain acetal (for example, chloroacetal), upon which the lower-boiling alcohol distills over leaving the desired cyclic acetal in good yield.

Using this method the following products have been prepared: chloroethylidene glycerol, -glycol, -trimethylene glycol; β -chloropropylidene glycerol and β -bromopropylidene glycerol.

Experimental

Preparation of 3, 5, 7-Trioxabicyclo [2, 2, 2] octane (A)

1, 3-Bromoethylidene glycerol 2-benzoate, the preparation of which is given in detail in a previous communication by the authors (7), m.p., 109° C., (40 gm.), was mixed with a solution of 4.5 gm. of potassium hydroxide in 100 cc. of water in a three-necked flask fitted with mercury seal stirrer and reflux condenser. The contents was stirred vigorously at 100° C. and 50% potassium hydroxide solution added at intervals as follows: after 2 hr. heating, 6 gm.; after 2½ hr. heating, 7.0 gm.; after 2½ hr. heating, 6.0 gm.; after 4 hr. heating, 6.0 gm.; after 5 hr. heating, 6.0 gm.

Solution of the benzoate took place after about an hour's heating and stirring. After all the potassium hydroxide had been added, the solution was heated for 30 min., then cooled and saturated with potassium carbonate. The precipitated potassium benzoate was filtered off, and both precipitate and filtrate extracted six times with ether. The ether extract was dried, and the ether removed, the residue solidifying completely. M.p., 99° C. Yield, 14.8 gm. (95%).

This solid gave a slight test for halogen. It was purified by heating with 25% potassium hydroxide under reflux at 100° C. for ten hours. Saturation with potassium carbonate and extraction with ether yielded a crystalline solid free from halogen. It recrystallized from warm ligroin (b.p., 60-70° C.) in ice-flower formation; m.p., 99° C. The solid possesses a high vapor pressure, a small sample placed on a watch glass disappearing completely in a few hours' time. It is very soluble in water, alcohol, ether, ethyl acetate, chloroform, toluene, benzene, glycol monomethyl ether and dioxane, less soluble in ligroin and carbon tetrachloride. Analysis:—Found: H, 6.8, 6.8; C, 51.6, 51.7%. Calcd. for $C_8H_8O_3$: H, 6.93; C, 51.68%. Mol. wt. (ethylene bromide). Found: 117, 115. Calcd. for $C_8H_8O_3$, 116.

The product gave no test indicating unsaturation and when treated with benzoyl chloride in pyridine solution gave no benzoate, the original substance being recovered unchanged.

Examination for Hydroxyl Groups with Magnesium Methyl Iodide Solution

Application of the method of Hibbert (5) indicated absence of any hydroxyl groups in the product. A weighed sample was dissolved in isoamyl ether and treated with an excess of methyl magnesium iodide in the same solvent, in a closed apparatus, in which the volume of any methane evolved could

be carefully measured; 0.2004 gm. (assuming one hydroxyl group per mole) should give 38.7 cc. of methane at N.T.P. Found, 1.7 cc., presumably due to a trace of moisture or impurity.

Hydrolysis of 3, 5, 7-Trioxabicyclo [2, 2, 2] octane (A)

This compound (5.056 gm.) was mixed with 20 cc. of 0.01 *N* sulphuric acid and the solution heated at 70° C. for ten hours. It was then cooled, almost neutralized with very dilute sodium hydroxide, and made up to a volume of 50 cc. (E). A small sample treated with ammoniacal silver nitrate gave a silver mirror immediately. The quantity of aldehyde group present was determined by the iodine oxidation method of Willstätter and Schudel (11). Analysis showed 95% of the theoretical quantity of aldehyde present.

Identification of Glycollic Aldehyde

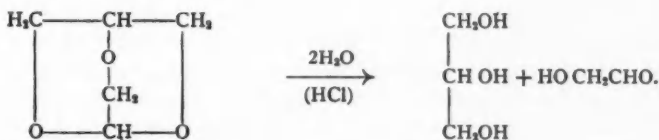
A sample of the original hydrolysis liquor (E) was added to a saturated alcoholic solution of *p*-nitrophenylhydrazine and the solution heated at the boiling point for a minute. A drop of concentrated hydrochloric acid was then added and the solution heated for a minute longer. It rapidly turned from orange to red, and a brick-red flocculent precipitate formed. The mixture was filtered, and the precipitate washed thoroughly with 50% alcohol and dried. It was insoluble in water, alcohol, ether, ligroin, ethyl acetate and chloroform, but soluble in pyridine, aniline, nitrobenzene, etc. It crystallized from pyridine in wine-red needles. These were filtered and washed thoroughly with ether. The dried, brick-red colored product gave a deep blue solution with alcoholic alkali. It softened at 290° C. and melted at 309-311° C. with evolution of gas, and was identical in properties with the *p*-nitrophenylosazone of glycollic aldehyde obtained by Wohl and Neuberg (12) and others (2, pp. 214-228; 9).

Isolation of Glycerol from the Hydrolysis Liquor (E)

The hydrolysis liquor (E, 40 cc.) was treated with 100 gm. of 5% sodium amalgam and the required amount of dilute sulphuric acid, the latter being added gradually over a period of two hours. The clear solution was poured off from the mercury, carefully neutralized, and this evaporated as completely as possible under reduced pressure (30° C. and 20 mm.). The residue was extracted with absolute methyl alcohol, the latter removed and the syrupy residue distilled (2 mm.). Most of the distillate (2 gm.) came over at 143-147° C., a small amount of charred product remaining in the flask. The fraction (b.p., 143-147° C./2 mm.) was then converted into the phenylurethane by heating one gram with four grams of phenyl isocyanate. The reaction mixture was heated on the steam bath for two hours, when crystallization took place and the whole mass became solid. This product was washed with ligroin to remove excess phenyl isocyanate and then dissolved in hot alcohol. On cooling and standing a white amorphous solid separated which was filtered off and dried (m.p., 181° C.). It could not be crystallized from any of the common solvents. The triphenylurethane of glycerol prepared

similarly was an amorphous, white solid, melting at 180° C. (from ethyl alcohol). A mixture of this with the previous product melted at 181° C.

The products of hydrolysis of 3, 5, 7-trioxabicyclo [2, 2, 2] octane are thus glycollic aldehyde and glycerol, hydrolysis at the acetal and ether linkages taking place simultaneously.



3, 5, 7-Trioxabicyclo [2, 2, 2] octane

Preparation of 3, 6, 8-Trioxabicyclo [3, 2, 1] octane (B)

(a) Preparation of Glycerol α -Benzoate

Glycerol α -benzoate was prepared according to the method of Fischer and Bergmann (3) with certain improvements resulting in a higher yield. Isopropylidene glycerol benzoate (100 gm.) (prepared by benzoylating isopropylidene glycerol) was mixed with 450 cc. of 0.075 *N* hydrochloric acid and stirred vigorously with an automatic stirrer at 55-60° C. until solution took place and the turbidity just disappeared. This required about two hours. The solution was immediately cooled, neutralized with potassium bicarbonate, saturated with sodium chloride and extracted repeatedly with ether. The ether extract was dried over anhydrous sodium sulphate. On distilling off the ether, the glycerol α -benzoate remained behind as an oil. After drying for some time in a vacuum desiccator over phosphorus pentoxide, a small sample was cooled with a solid carbon dioxide ether mixture when crystallization took place. The main portion of the oil was seeded with these crystals and rapidly solidified. The solid glycerol α -benzoate was recrystallized from a solvent consisting of 99% of anhydrous ether and 1% of petroleum ether; m.p., 36° C. Yield, 81.5 gm. (98%). The highest yield obtained by Fischer and Bergmann was 63%.

(b) Preparation of 1, 2-Bromoethylidene Glycerol 3-Benzoate

Crystalline tribromoparaldehyde was prepared in 15% yield by the bromination of paraldehyde, following, in general, the procedure of Stepanow (10) but using a few drops of concentrated sulphuric acid as a catalyst, and allowing the reaction mixture to stand at 0° C. for several days. The tribromoparaldehyde, after recrystallization from alcohol and ligroin (b.p., 80-90° C.) melted at 103-104° C.

To 101 gm. of pure crystalline tribromoparaldehyde was added 182 gm. of pure crystalline glycerol mono α -benzoate and 12 drops of 40% sulphuric acid, and the mixture heated at 90-110° C. for 24 hr. It became homogeneous and liquid upon short heating, and at intervals of about four hours a vacuum was applied to the flask to remove water formed in the reaction. The reaction mixture was cooled, washed several times with water, extracted with ether,

the ether extract washed twice with dilute sodium hydroxide solution, once with concentrated sodium bisulphite solution, three times with water, and then dried over anhydrous sodium sulphate. After removal of solvent, distillation of the residual liquid yielded 130 gm. (52%) of 1,2-bromoethylidene glycerol 3-benzoate; b.p., 169-171° C./1 mm. n_D^{20} , 1.5449.

(c) *Preparation of 3, 6, 8-Trioxabicyclo [3, 2, 1] octane (B)*

Experiment 1. Pure 1,2-bromoethylidene glycerol 3-benzoate (50 gm.) was mixed with a solution of 5 gm. of potassium hydroxide in 200 cc. of water in a three-necked flask fitted with a mercury seal stirrer and reflux condenser and the product stirred vigorously at 100° C. for two hours. Further quantities of 3 gm. of potash in 5 cc. of water were then added after intervals of 2, 4, 6 and 7 hr. respectively. After a total of 15 hr. of stirring at 100° C., the contents of the flask was cooled, saturated with potassium carbonate and extracted repeatedly with ether. The ether extract was dried over anhydrous sodium sulphate, the ether removed and the residue fractionated. Two products were obtained:—

(a) A substance distilling at 68-70° C./12 mm. and which solidified on cooling; m.p., 56-57° C. Yield, 2 gm. (10%).

(b) A substance distilling at 141-142° C./12 mm. n_D^{25} , 1.5006. Its properties corresponded with those of 1,2-bromoethylidene glycerol as found in a previous investigation (7). Yield, 24 gm. (75%).

The recovery of such a large amount of the acetal indicates its comparative inertness towards alkali as compared with the isomeric 1,3-bromoethylidene glycerol.

Experiment 2. In order to ascertain whether the yield of bicyclic product could be increased, a second experiment was performed with more concentrated alkali using 75 gm. of the 1,2-bromoethylidene glycerol 3-benzoate and a solution of 13 gm. of potassium hydroxide in 150 cc. of water. The mixture was stirred vigorously at 100° C. for one hour and two lots of 13.5 gm. each of potash dissolved in 15 cc. of water added after one hour, and three hours' heating respectively, followed by a third and fourth addition of 10 gm. of potash dissolved in 10 cc. of water after 4½ and 5½ hr. respectively.

At the end of six hours the oily benzoate had completely dissolved. Heating at 100° C. was then continued for an additional 13 hr. The solution was cooled, saturated with potassium carbonate, the precipitated potassium benzoate filtered off, and both precipitate and filtrate extracted repeatedly with ether. The ether extract was dried and the ether removed. The solid residue left was then distilled very rapidly directly into a flask cooled by running water. Under these conditions the entire residue distilled at 68-70° C./12 mm. yielding 9 gm. of a colorless crystalline solid; m.p., 56-57° C. Yield, 31%. No 1,2-bromoethylidene glycerol was obtained as in the previous experiment. About 60 or 70% of the reaction products were ether insoluble

and remained in the alkali liquor, unextracted by the ether. Extraction with alcohol was impracticable due to the presence of large quantities of potassium benzoate.

Experiment 3. A third experiment was conducted under the same conditions as above, but using 1, 2-bromoethylidene glycerol (*i.e.*, the hydrolyzed product) instead of 1, 2-bromoethylidene glycerol 3-benzoate. The reaction product was neutralized with 10% sulphuric acid, and evaporated to dryness under reduced pressure. The bicyclic product formed in the reaction was lost at this point due to its high volatility in presence of water vapor. The residue was extracted with alcohol. After removal of the latter a viscous syrup was left which contained no halogen. On distillation under reduced pressure a portion distilled over a wide range (135-175° C./3 mm.) with some decomposition; the residue, a semisolid resinous mass, could not be distilled. This was not investigated further.

The white crystalline solid (m.p., 56-57° C.) obtained from the ether extracts in experiments Nos. 1 and 2 was entirely free from halogen. It showed a similar behavior towards organic solvents as the previous isomer (A). It was saturated, contained no hydroxyl groups and was readily volatile, small quantities left exposed to the open air disappearing in a short time (one or two hours). Analysis:—Found: H, 6.8, 7.0; C, 51.5, 51.7%. Calcd. for $C_8H_{10}O_3$: H, 6.93; C, 51.68%. Mol. wt. (ethylene bromide):—Found: 118, 120. Calcd. for $C_8H_{10}O_3$, 116.

Hydrolysis of 3, 6, 8-Trioxabicyclo [3, 2, 1] octane (B)

This product (m.p., 56-57° C.) was hydrolyzed exactly as in the case of the corresponding isomer (A). Glycollic aldehyde and glycerol were identified as the products of hydrolysis.

The action of aqueous alkalis on the isomeric β -chloropropylidene glycerols as well as on the corresponding α , β -dibromo compounds is being investigated. The latter investigation will be of interest as indicating a new application of the Hibbert-Michael ring partition principle.

NEW METHOD FOR THE SYNTHESIS OF CYCLIC ACETALS

Preparation of Chloroethylidene Glycerol

Anhydrous glycerol (30 gm.) and 50 gm. of chloroacetal were heated together with stirring at 115° C. In 10 or 15 min. the mixture had become homogeneous, and a short fractionating column and condenser were then attached to the flask and the temperature gradually raised to 160° C. Ethyl alcohol distilled over (b.p., 78° C.). Yield, 26.5 gm. (88% of theory). The residual oil was distilled under reduced pressure. Yield of chloroethylidene glycerol, 40 gm. (80%); b.p., 130-134° C./13 mm. Analysis:—Calcd. for $C_8H_9O_2Cl$; Cl, 23.02%. Found: 23.21%. In a similar manner chloroethylidene glycol (b.p., 57° C./13 mm.), chloroethylidene trimethylene glycol

(b.p., 60-62° C./11 mm.), β -chloropropylidene glycerol (b.p., 129-135° C./15 mm.) and β -bromopropylidene glycerol (b.p., 136-140° C./18 mm.) were prepared in yields of 40, 60, 56 and 65% respectively.

The method is being applied to the synthesis of other cyclic acetals and its use for determining the structure of carbohydrates and polysaccharides is under investigation.

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THE CONDENSATION OF CERTAIN γ -KETONIC ESTERS WITH AROMATIC ALDEHYDES. II¹

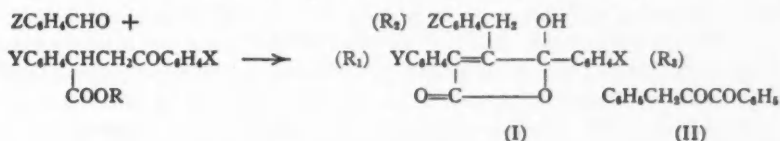
By C. F. H. ALLEN,² G. F. FRAME,³ J. B. NORMINGTON⁴
AND C. V. WILSON⁴

Abstract

The condensation of benzaldehyde with methyl and ethyl α -aryl- β -benzoyl propionates in the presence of sodium methylate, followed by acidification, gave unsaturated ketric acids. This result was unexpected in view of the previously described similar work in which lactols were formed; the only difference in the molecules is the absence of a substituent group in the benzoyl radical of the ester.

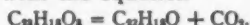
The structure of the acids was carefully determined. On being heated they lost carbon dioxide, forming α , β -unsaturated ketones. One of the latter added hydrogen chloride to give two stereoisomeric saturated chloroketones, which were also synthesized by an independent method.

In an earlier paper (1) several lactols (I) made by alkaline condensation of certain γ -ketric esters and aromatic aldehydes were described. These lactols were alike in that all had a substituent group in the para position of the third benzene ring (R_3). ($X = \text{Cl, Br, OCH}_3$; $Y, Z = \text{H}$)



When efforts were made to prepare an unsubstituted lactol ($X, Y, Z = \text{H}$) it was found that the product (A) exhibited noticeable differences in chemical properties. In the Grignard machine it showed one active hydrogen and addition of one mole of reagent, while the lactols previously described added two moles of reagent to one active hydrogen.

The substance (A) lost carbon dioxide on being heated, the pyrolysis proceeding in accordance with the equation



This was, indeed, surprising because the lactols did not exhibit this behavior, being unchanged until heated much higher above their melting points and then decomposing completely. At first it was thought that the substance was a β -lactone, although the latter type of compound is not regenerated (as was ours) by acidifying solutions of its salts. Recently (3) it has been shown that in a few instances β -lactones can be formed by acidification of alkali salts, so the fact that our substance was easily recovered on similar treatment did not exclude that type of structure. The pyrolysis product

¹ Manuscript received January 5, 1933.

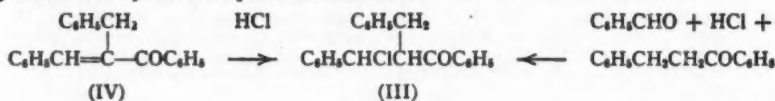
Contribution from the Department of Chemistry, McGill University, Montreal, Canada, with financial assistance from the National Research Council of Canada.

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formed an oily oxime and phenylhydrazone, but gave a crystalline mono-2, 4-dinitrophenylhydrazone, showing the presence of one carbonyl group. On ozonization benzaldehyde was produced. By ozonizing a substance made by pyrolysis of a homologue (B) having a methoxyl group in ring R_1 (Formula I: $Y = OCH_3$, $Z, X = H$) anisaldehyde was obtained, indicating the presence of $R_1CH=$ and showing which R it was. It added hydrogen chloride to form a mixture of the two stereoisomeric chloroketones (III), which were synthesized by an independent method.

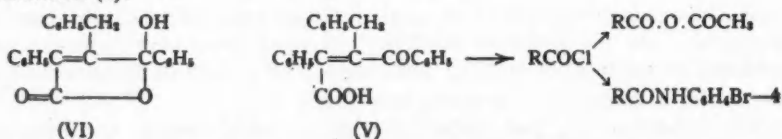


Therefore the pyrolysis product must be α -benzylbenzalacetophenone (IV).

The substance (A) readily formed an oxime and resembled the lactols previously described on oxidation; while permanganate was useless, chromic acid gave an α -diketone, in this case phenyl benzyl diketone (II). This pointed to an essentially similar structure, since when a homologue (B) was prepared with R_1 tagged by a methoxyl group ($Y = CH_3O$) it also gave phenyl benzyl diketone, showing that R_1 was not involved. The formation of this diketone excluded any of the possible β -lactone structures.

It reacted with thionyl but not acetyl chloride; since the product gave *p*-bromoanilide and a mixed acet-anhydride it must have been an acid chloride.

Collectively these properties point to the structure of an open chain γ -ketonic acid (V) for the substance (A); there was no evidence of the isomeric lactol (VI) as was anticipated in view of the results previously described (1).



It is difficult to find any connection between the presence or absence of a substituent group in the para position of only one ring, and the formation of a cyclic structure in one instance and not in another. It would appear that the substituent must activate the carbonyl group for some, as yet, unexplained reason. Buck and Ide (2) have noted a comparable case of activation of the carbonyl group in *o*-chlorobenzil, the $\text{C}=\text{O}$ next the chlorine-containing phenyl group being the more reactive—the steric effect was expected to make it less active. The authors are continuing this work using compounds with more varied groups.

Experimental

A. Preparation of the Esters

The methyl and ethyl esters of α -phenyl- β -benzoyl propionic acid were made by refluxing the acid in the appropriate alcohol in the presence of sulphuric acid for four hours. The methyl ester (m.p., 103°C .) was obtained

in a yield of 96%, and the ethyl ester (m.p., 41° C.) in a yield of 71%. The methyl α -(*p*-anisyl)- β -benzoylpropionate was made by the method as outlined in the previous paper (1, ref. 18) from anisalacetophenone; the yields were: nitrile, 93%; acid, 91%; ester, 95% (m.p., 105° C.).

B. Condensation of the Esters with the Aldehydes

This was done by the procedure previously described, except that it was found that the alcohol did not have to be absolute.

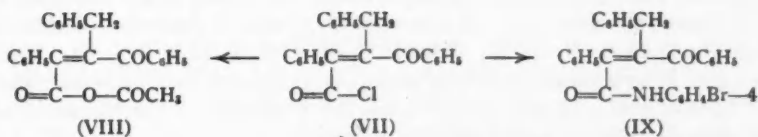
α -Phenyl- β -benzoyl- β -benzyl acrylic acid (V), m.p., 145°C., was very soluble in the usual organic solvents except petroleum ether, and crystallized in plates. The yield was 85% from the methyl ester and 87% from the ethyl ester. It evolved carbon dioxide from warm sodium bicarbonate solution and was precipitated unchanged on addition of acid. The corresponding methoxy homologue, α -(*p*-anisyl)- β -benzoyl- β -benzyl acrylic acid (B) melted at 136° C. The use of anisaldehyde, piperonal and *p*-chloro-benzaldehyde gave thick oily acids. Analyses: Calcd. for (V) $C_{23}H_{18}O_3$: C, 80.7; H, 5.2%; (B) $C_{24}H_{20}O_4$: C, 77.4; H, 5.4%. Found: (V) C, 80.4; H, 5.2%; (B) C, 77.2; H, 5.3%. In the Grignard machine it reacted with two moles of reagent, liberating one mole of gas; this indicated one active hydrogen and one addition. The methyl ester, prepared through the silver salt, and from the acid chloride, was oily and was not analyzed.

The oxime. This was prepared in the usual way and recrystallized from *n*-butyl alcohol; it formed small plates (m.p., 181° C.) sparingly soluble in hot methyl and ethyl alcohols, but moderately soluble in ether. Analysis: Calcd. for $C_{23}H_{19}O_2N$: N, 3.9%. Found: N, 3.7%.

C. Oxidation

By the method described in the earlier paper, the acid (V) was oxidized by chromic acid in acetic acid at 40° C., 4.6 gm. giving 2.7 gm. of phenyl benzyl diketone. The latter was identified by conversion into the quinoxaline, m.p. 97° C.,—a mixed melting point with a sample at hand was not depressed. The diketone gave a deep violet-brown color with alcoholic ferric chloride. The methoxy acid (B) on similar treatment gave anisic acid (identified by mixed melting point) and phenyl benzyl diketone.

D.



The acid chloride (VII). The acid (V), 4 gm., and 15 cc. of thionyl chloride was refluxed on a water bath for a half hour, and the solvent allowed to evaporate spontaneously. Long silky needles separated. The halide was purified by recrystallizing several times from ether; m.p. 79° C., turning pink. Analysis: Calcd. for $C_{23}H_{17}O_2Cl$: Cl, 9.8%. Found: Cl, 9.5, 9.7%. The acid was recovered unchanged after refluxing a half hour with acetyl chloride (cf. Ref. 1, p. 611).

The acet-anhydride (VIII). A mixture of 2.4 gm. of the chloride, 2.2 gm. of silver acetate and 20 cc. of absolute ether was refluxed for an hour. After filtering from silver salts the anhydride separated in practically a quantitative yield. On recrystallizing from methyl alcohol it formed dense white prisms; m.p., 126° C. Analysis:—Calcd. for $C_{23}H_{20}O_4$: C, 78.1; H, 5.2%. Found: C, 78.1; H, 5.2%. The same anhydride was also prepared by warming for five minutes 1 gm. of the acid (V) in 10 cc. of acetic anhydride containing a trace of sulphuric acid; it crystallized out after standing a half hour. The acid was regenerated on hydrolysis.

The p-bromanilide (IX). The chloride (2 gm.) and two equivalents (2 gm.) of *p*-bromaniline in 35 cc. of benzene was refluxed for an hour and filtered from the separated *p*-bromaniline hydrochloride. The anilide that remained on partial removal of the solvent was recrystallized to constant melting point from acetone. It formed microscopic needles; m.p., 179° C. Analyses:—Calcd. for $C_{23}H_{22}O_2NBr$: N, 2.8; Br, 16.1%. Found: N, 2.6; Br, 16.3%. The *p*-bromanilide was at first recrystallized from benzene, but it separated with solvent of crystallization. It is insoluble in methyl and ethyl alcohols and cyclohexane, moderately soluble in *n*-propyl alcohol, but easily dissolves in acetone and benzene.

The *p*-iodoanilide was made in essentially the same way, and purified by dissolving in hot benzene and adding an equal volume of cyclohexane; m.p., 200° C. It also separated with benzene of recrystallization. Analysis:—Calcd. for $C_{23}H_{22}O_2NI \cdot C_6H_6$; I, 20.5%. Found: I, 20.7%. Both the anilides gave a red-violet color with concentrated sulphuric acid.

E. Pyrolysis

The acid (V), 137 gm., in a 250-cc. Claisen flask was heated at 230-240° C. for 2½ hr. and the resulting oil distilled *in vacuo*: 114 gm. (95.6% of the theoretical amount) of clear yellow oil was obtained; b.p., 238-250° at 6 mm. After several days it began to solidify. A few crystals were removed for seeding and the remainder of oil and solid dissolved in a minimum amount of warm methyl alcohol; on inoculating with the sample the whole slowly crystallized. When pure it formed white prisms; m.p., 48° C. It is very soluble in the usual organic solvents except petroleum ether but does not crystallize well from any except methyl alcohol. Analysis:—Calcd. for $C_{22}H_{18}O$: C, 88.6; H, 6.0%. Found: C, 88.2, 88.2; H, 6.0, 5.9%. Unless very pure it slowly hydrolyzes, becoming very oily and forming benzaldehyde and benzoic acid. In the Grignard machine it evolved no gas but consumed one mole of reagent, indicating one addition.

The oxime and phenylhydrazone were oils. The 2, 4-dinitrophenylhydrazone, prepared in the usual way, formed carmine needles from chloroform-methyl alcohol solution; m.p., 189° C. Analysis:—Calcd. for $C_{23}H_{12}O_4N_4$: N, 11.7%; Found: N, 11.1%.

Permanganate oxidation of the ketone gave practically the calculated amount of benzoic acid. Chromic acid gave mostly benzoic acid but also

a little oil that probably contained phenyl benzyl diketone, since it gave the brown-violet color with ferric chloride. After ozonization (ozone destroys the diketone) of the pyrolysis product benzaldehyde was obtained, separated by steam distillation and identified by preparation of the phenylhydrazone; m.p., 155° C.

The methoxyketone was obtained only as a thick, viscous, colored oil that had not crystallized after two years; on ozonization as above it gave anisaldehyde. The residual oil gave a ferric chloride test for phenyl benzyl diketone but none could be isolated. The methoxyketone gave an oily 2, 4-dinitrophenylhydrazone.

α -Benzyl- β -chloro- β -phenylpropio-phenone (III). (a) A solution of 2 gm. of the unsaturated ketone (IV) in 10 cc. of absolute methyl alcohol was cooled in a freezing mixture while being saturated with hydrogen chloride. After standing in a cold place for two days the white solid that had separated was filtered. It melted over a range, 101-105° C., and was obviously a mixture. After many tedious and fruitless attempts to separate the isomers by fractional crystallization and precipitation from ordinary solvents, a solution in cyclohexane was allowed to evaporate to dryness very slowly. Two kinds of crystals were evident; the larger ones were picked out by hand and when purified formed long narrow plates; m.p., 126° C. The smaller crystals left were then recrystallized several times from methyl alcohol and the stereoisomer obtained in the form of fine needles; m.p., 132° C. Analyses:—Calcd. for $C_{23}H_{19}OCl$; Cl, 10.6%. Found: (126° C.) Cl, 10.4; (132° C.) Cl, 10.4%. The least soluble component of solutions of the mixed chlorides is a mixture that melts at 108° C. and after this has separated very little material remains. The chlorides are insoluble in petroleum ether, slightly soluble in the alcohols and cyclohexane, moderately soluble in ether, and very soluble in chloroform, acetone, and benzene.

(b) *Synthesis.* A mixture of the stereoisomers was prepared by the method of Kohler and Nygaard (4) for a similar chloroketone, from benzaldehyde, benzylacetophenone and hydrogen chloride. The solid obtained was triturated with ether and filtered; 15 gm. of the ketone gave 13.5 gm. of the mixed chloroketones, which was separated as above and found to be identical in crystal form, melting point, and mixed melting point.

Acknowledgment

This work has been assisted by a generous grant from the Cyrus M. Warren Fund of the American Academy of Arts and Sciences.

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NOTE: In the earlier paper (1, p. 612) the melting point of the methyl ester was accidentally omitted. It is 78° C., and the melting point when mixed with the isomeric methyl ester is 58-63° C.

THE ALKALOIDS OF FUMARACEOUS PLANTS

III. A NEW ALKALOID, BICUCULLINE, AND ITS CONSTITUTION¹

BY RICHARD H. F. MANSKE²

Abstract

A base, now named bicuculline, previously isolated from *Dicentra cucullaria* and referred to as alkaloid α has been obtained from *Corydalis sempervirens* and *Adlumia fungosa*. A chemical examination of this alkaloid has disclosed an exceptionally close relation to hydrastine, from which it differs only by the substitution of a methylenedioxy group for the two methoxyl groups. Accordingly, hydrolytic oxidation yields hydrastinine and 2-carboxy-3:4-methylenedioxy-benzaldehyde, which on reduction is converted into the known 3:4-methylenedioxy-phthalide.

In a previous communication, the author (5) recorded the isolation of a base, $C_{20}H_{17}O_4N$ from *D. cucullaria*, which was referred to as alkaloid α . The quantity obtained at that time was insufficient for a detailed chemical investigation, but recently a further supply has become available in the course of a chemical examination of *Corydalis sempervirens* Pers. and of *Adlumia fungosa* Greene, accounts of which it is proposed to publish in due course.

Since alkaloid α has proved to be a distinct substance, it is proposed to name it *bicuculline*, indicative of its botanical origin, the term *Bicuculla* being used by some botanists for the genus *Dicentra*. In connection with its isolation from *A. fungosa*, it may be pointed out that Schlotterbeck (7) obtained from the same plant (*A. cirrhosa* Raf.) a small amount of an alkaloid melting at 176-177° C. Although no analyses are recorded, there can be little question of identity, and the same is probably true of an alkaloid (m.p. 175° C.), regarded as phenolic, isolated from *Corydalis decumbens* by Asahina and Motigase (1) and also not analyzed. It is furthermore possible that bicuculline may have been isolated from other sources without having been recognized as such, on account of a well-defined dimorphism which it exhibits. In one case, it was observed that the base had resolidified in a melting point tube and when the melting point was again determined it had increased from 177° C.* to 193-195° C. At about the same time, an alkaloid melting at 196° C. had been isolated from *A. fungosa*, and inasmuch as bicuculline had been previously isolated from the same plant, identity was immediately suspected.

A supersaturated solution of bicuculline (m.p. 177° C.) in methanol when seeded with a crystal of m.p. 196° C., rapidly deposited the latter form. Furthermore, the hydrochlorides prepared from both forms had the same appearance and melted alone or admixed at 259° C. to a reddish yellow liquid.

Although bicuculline is not precipitated when its solution in acid is poured into an excess of a hot aqueous potassium hydroxide, it was soon recognized that its behavior was more like that of a lactone than that of a phenol. An alcoholic solution of the base when treated with aqueous alkalis, gave an immediate precipitate which dissolved only with considerable difficulty.

If it be assumed that one lactonic group is present, only four more oxygen atoms need be accounted for and, since methoxyl is absent, the presence of

*All melting points are corrected.

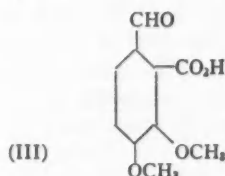
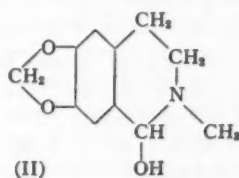
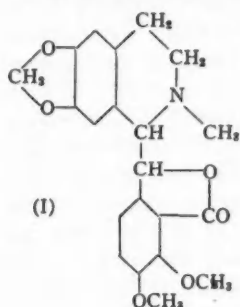
¹ Manuscript received January 16, 1933.

² Contribution from the National Research Laboratories, Ottawa, Canada.

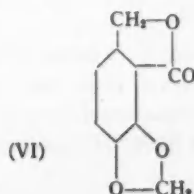
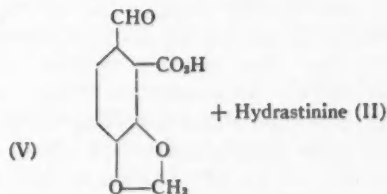
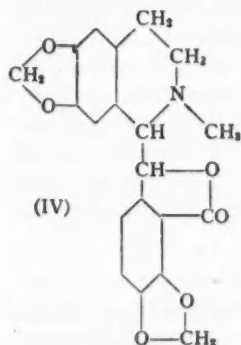
³ Associate Research Chemist, National Research Laboratories, Ottawa.

two methylenedioxy groups would suffice. Finally, if the alkaloid be regarded as an isoquinoline derivative on account of its associates, the conclusion is incumbent that it is closely related to hydrastine, $C_{21}H_{21}O_6N$, from which it differs by CH_4 , *i.e.*, the substitution of a methylenedioxy group for two methoxyl groups. In confirmation of this supposition, it yields the same fluorescent solution that hydrastine gives when heated with manganese dioxide in dilute sulphuric acid.

Recently, Hope, Pyman, Remfry and Robinson (2, p. 244) have described an elegant procedure for the hydrolytic oxidation of hydrastine (I) to yield hydrastinine (II) and opianic acid (III), and when the same conditions are observed in the case of bicuculline (either form) hydrastinine is obtained in nearly quantitative yield.



The acidic fragment was readily isolated from the hydrastinine filtrate, and proved to be an ortho aldehyde acid, treatment with hydroxylamine in hot solution yielding a substituted phthalimide. While these observations yielded no information as to the position of the methylenedioxy group, in the lower nucleus, the precedents of hydrastine and narcotine amply justify formula (IV) for bicuculline.



The aldehydic acid on this basis should have formula (V). This appears to be a new substance, and in fact represents the first example of a methylenedioxy analogue to the well-known opianic acids, attempted syntheses of which have failed (6, 9). Two of the possible methylenedioxy-phthalides are, however, known (6, 9), the substance (VI) having been synthesized by Perkin and Trikojus (6) and later confirmed by Späth and Holter (8, p. 1897). Opianic acid is readily reduced to meconine with sodium amalgam and when the similar reduction was applied to the aldehydic acid from bicuculline, a lactone was obtained which, after repeated recrystallization from water, melted at 234° C. (227° C. uncorr.). It is obviously identical with the substance (VI) which melts at 227° C. (? corr.) (8, p. 1897). The alternative 4 : 5-methylenedioxy-phthalide melting at 189° C. (9) is excluded, so that formula (V) applies to the acidic fragment of the hydrolytic oxidation and consequently formula (IV) completely represents bicuculline.

In conclusion, it may be of interest to point out that alkaloids of the general nuclear structure of hydrastine have been obtained from plants of three different natural orders, namely, Ranunculaceae (hydrastine), Papaveraceae (narcotine), and Fumariaceae (bicuculline), but in each case the phytochemical processes have been subjected to a subtle variation. The analogy, however, goes even further. Both *Hydrastis canadensis* and *Papaver somniferum* contain appreciable amounts of meconine. In the case of *D. cucullaria*, the corresponding methylenedioxy-phthalide (VI) has been also isolated, but whether this is to be regarded as a decomposition product of bicuculline or present as such in the plant is still a matter of doubt.

Experimental

Hydrolytic Oxidation of Bicuculline—Isolation of Hydrastinine (II)

One gram carefully purified bicuculline (m.p. 177° C.) was dissolved in a mixture of 2 cc. of concentrated nitric acid and 8 cc. of water and heated on a steam bath for 20 min. The yellow solution was thoroughly cooled and treated with pellets of potassium hydroxide, one at a time with cooling, until it was strongly alkaline and crystallization of hydrastinine was complete. The solid was filtered off, washed with cold water and dried *in vacuo*. Yield, 0.5 gm. The substance, as thus obtained, was quite pure and melted sharply at 117° C. Treatment in methanol with nitromethane yielded the characteristic addition product, melting sharply at 121-122° C. (uncorr.), first described by Hope and Robinson (3, p. 2136) and stated to melt at the same temperature (? corr.). An aqueous solution of the hydrochloride exhibited an intense greenish-blue fluorescence. With bicuculline of m.p. 196° C. the same products were obtained.

Isolation of 2-Carboxy-3 : 4-methylenedioxy-benzaldehyde (V)

The alkaline filtrate from the hydrastinine was acidified with dilute sulphuric acid and thoroughly extracted with ether. The combined extract was washed with a little water, dried over sodium sulphate and most of the

ether distilled off. The residue was completely freed of solvent and a trace of nitric acid *in vacuo* over potassium hydroxide; yield, 0.5 gm. The crystalline product was dissolved in a little hot water, filtered with the aid of charcoal and cooled, when crystallization readily ensued. The 2-carboxy-3:4-methylenedioxy-benzaldehyde thus obtained melted not quite sharply at 155° C. and gave on gentle warming with phenol and concentrated sulphuric acid, a deep cherry red color which slowly changed to an intense orange.

The dual aldehydic and ortho-carboxylic nature of the substance is shown by the fact that it yields, on treatment with hot alcoholic hydroxylamine acetate, a colorless crystalline substance which is obviously the imide of the corresponding orthodicarboxylic acid. The imide is sparingly soluble in cold water or methanol and when heated in a melting point tube largely sublimes before it melts indefinitely, at about 270° C. When heated with strong alkali on the steam bath, it rapidly dissolves and ammonia is liberated.

Reduction of the Aldehydic Acid to the Phthalide (VI)

An aqueous solution of the substance was acidified with dilute sulphuric acid and treated with sodium amalgam in large excess. When all the sodium had been used up, the aqueous solution was separated from the mercury, freed of a turbidity by means of charcoal and then exhausted with ether. The residue from the ether solution was recrystallized twice from hot water and then consisted of colorless flat elongated plates which melted at 232-233° C. (227° C. uncorr.), and sublimed to the cooler part of the tube. Späth and Holter give the melting point of sublimed 3:4-methylenedioxy-phthalide as 227° C. (? corr.). The substance dissolved readily in warm potassium hydroxide solution. Calcd. for $C_9H_6O_4$: C, 60.67; H, 3.37%. Found: C, 60.48; H, 3.55%.

3:4-Methylenedioxy-N-ethylphthalimide

While the constitution of the aldehydic acid has been sufficiently demonstrated, it seemed nevertheless desirable to approach the matter from another direction. For this purpose, the substance was oxidized in the cold with aqueous potassium permanganate. The filtrate from the manganese dioxide was acidified with sulphuric acid and exhausted with ether. The crystalline residue from the extract was treated with excess ethylamine in aqueous solution, and evaporated to dryness. The residue was distilled *in vacuo* and recrystallized from methanol. It crystallized in colorless needles melting at 130° C. Späth and Holter (8) give 128° C. (? corr.).

N-Methyl-bicuculleine

Since the methohydroxides of hydrastine and of narcotine on appropriate treatment yield substances of which narceine is the best-known representative, it seemed desirable to effect the analogous reaction in the case of bicuculline by Hope and Robinson's (4) method.

One gram of bicuculline in chloroform-methanol was heated under reflux for two hours with an excess of methyl iodide. The solvents were distilled

off and the residue in hot aqueous solution was converted into the methochloride by means of freshly precipitated silver chloride. The colorless filtrate was basified with excess potassium hydroxide in the cold and the orange quaternary base filtered off and washed with cold water. It was then suspended in water and boiled for about 30 min. The undissolved yellow solid was filtered off and boiled again for 30 min. with water. Further treatment failed to change or dissolve this product appreciably. The combined filtrates were evaporated to a small volume. In the course of a short while, colorless plates, melting at 246°C ., crystallized from the cooled solution. The yield was only 0.1 gm. After being twice recrystallized from hot water, N-methylbiccuculleine was obtained in fragile colorless plates melting at 250°C . to a brown melt which subsequently effervesced. Calcd. for $\text{C}_{21}\text{H}_{21}\text{O}_7\text{N}$; C, 63.16; H, 5.26; N, 3.51%. Found: C, 62.45; H, 5.40; N, 3.62%.

Biccuculline and its Hydrochloride

The lower melting form (177°C .) was the first to have been isolated and may be obtained by seeding a cold supersaturated methanolic solution of the base with this form. The higher melting form (196°C .) frequently crystallizes when a concentrated methanolic solution is vigorously boiled. The concentrated methanolic solutions are conveniently prepared by evaporating a chloroform solution of the alkaloid to a resin and dissolving the latter in hot methanol.

Biccuculline hydrochloride is readily prepared by neutralizing a chloroform solution with alcoholic hydrogen chloride and evaporating repeatedly with chloroform to remove water and alcohol. The cooled concentrated solution generally deposits colorless irregular plates. Cautious addition of ethyl acetate facilitates filtering and washing. The salt melts at 259°C . with but little immediate decomposition. It is readily soluble in hot water and crystallizes from the cooled solution if seeded and particularly if concentrated hydrochloric acid is added.

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INHERITANCE OF BUNT AND LOOSE SMUT REACTION AND OF CERTAIN OTHER CHARACTERS IN KOTA X RED BOBS AND GARNET CROSSES¹

BY THOMAS KILDUFF²

Abstract

Genetic studies were made in F_2 , F_3 and F_4 on the progeny of two common wheat crosses, Kota X Red Bobs and Kota X Garnet. Two chlamydospore collections from pure line hosts were used for inoculation in the bunt studies. The heritable nature of bunt reaction was clearly demonstrated in these crosses and evidence obtained that the degree of resistance found in Garnet must be conditioned by a single major factor. No genic hypothesis could be offered to explain the findings in regard to loose smut reaction due to a lack of agreement in infection percentages in the F_2 and F_4 . Red kernel color in Kota and Red Bobs was found to be governed by two factor pairs. The results on the inheritance of awn characters agreed with the earlier findings of Clark and other workers with crosses of similar awned types, several factor pairs being involved. Two main factors appeared to condition the difference in strength of straw between Kota and Garnet, but the entire expression of the character can best be explained on a multiple factor basis. Correlated inheritance studies, made with the use of the correlation ratio η , indicated the presence of two linkage groups concerned with reaction to the bunt forms used. However, the linkage apparent between strength of straw and bunt susceptibility factors and between awn type and bunt resistance factors is not considered to be close enough to interfere with breeding for desirable bunt resistant varieties.

Introduction

Attention has been drawn recently by Aamodt (1) to the need in western Canada for improved varieties of hard red spring wheat resistant to those diseases which are likely to be destructive and limiting factors in production. The wheat improvement program at the University of Alberta considers not only resistance to bunt, loose smut, foot rots, drought and lodging, but keeps to the fore consideration of such important agronomic characters as earliness, quality and high yielding ability. The program also includes basic genetic studies on wheat plant characters, both morphologic and physiologic. The study here reported was undertaken, as part of the more general program, to ascertain the mode of inheritance of reaction to bunt and loose smut in certain common wheat crosses and to discover if possible any relation existing between disease reaction and the other characters studied, namely, seed color, awning and strength of straw.

Materials

Parental Varieties

Three varieties of hard red spring wheat, *Triticum vulgare*, Host., were used as parents in the two crosses studied. All three, namely, Kota, Red Bobs, and Garnet, are good yielders and fairly well adapted to central Alberta.

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Contribution from the Department of Field Crops, University of Alberta, Edmonton, Canada, with financial assistance from the National Research Council of Canada. This work is in part from a thesis presented to the Committee on Graduate Studies, University of Alberta, in partial fulfilment of the requirements for the degree of Master of Science.

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Kota was originally introduced into North Dakota from Russia by H. L. Bolley in 1903, but it was not produced on a commercial scale until 1919. It is a midseason variety, moderately drought resistant and resistant to many physiologic forms of *Puccinia graminis tritici* in the field, but it is susceptible to bunt and loose smut. The mid-dense, fusiform head bears strong awns 30 to 80 mm. in length. Not only are there long awns on the lemmas or flowering glumes, but the beak of the outer glume is elongated 3 to 20 mm. to an awnlike point. The straw is somewhat weak and under very favorable growing conditions shows a distinct tendency to lodge (10). Kota is not a good milling and bread-making wheat.

Red Bobs originated from a red-kernelled head selection made in 1910 by Seager Wheeler, Rosthern, Saskatchewan, from a field of White Bobs. It was distributed commercially in 1918. Red Bobs is an early to midseason variety, not particularly drought-resistant and susceptible to stem rust. It proved to be immune to the collection of loose smut used in these experiments. The oblong, mid-dense head is sometimes clavate, is entirely without awns, or awnlets, and has beaks wanting to 0.5 mm. long. The straw of this variety is moderately strong, standing up fairly well under most conditions (10). Red Bobs is a good milling wheat and its flour is nearly equal in quality to that of Marquis.

Garnet, a comparatively recent (1926) entrant to the group of commercially important wheats, is a production of the Central Experimental Farm, Ottawa. It is from a Preston A \times Riga M cross made by Chas. Saunders in 1905. This variety is considerably earlier than Red Bobs, but is also very susceptible to stem rust. In these tests it proved to be resistant to bunt and loose smut. The fusiform mid-dense head shows considerable variability in regard to the length and distribution of the awnlets. Some heads are almost bald, some bear a few short apical awnlets (2 to 5 mm.) only, while the majority have awnlets (2 to 10 mm.) well distributed down the spike. The beaks are about 1 mm. long. The straw is fine and mid-strong, under some conditions showing a tendency toward weakness. Garnet is discriminated against by some millers and bakers on account of the lower quality and the distinctly yellow tinge in the flour it produces (31, 35).

Crosses

In 1929, a number of F_2 bulk crosses were received from J. A. Clark, of the Division of Cereal Crops and Diseases, U.S.D.A., Washington, D.C. Of these, two separate populations of a Kota \times Garnet cross (No. 109 and No. 112) and two of a Kota \times Red Bobs cross (No. 115 and No. 118) were turned over to the writer for the present study.

Bunt Inoculum

Two chlamydospore collections of *Tilletia*, which had been grown for two years (1928-29) on pure hosts in the University varietal plots, were used for inoculating the F_2 plants. The inoculum referred to in this study as "Kota bunt" was obtained from bunted heads of the Kota variety and that as

"Red Bobs bunt" from Red Bobs (University of Alberta No. 222). A chlamydospore collection was taken from the parental check rows of Kota which had been inoculated with Kota bunt in 1930 and was used for inoculating those lines tested in F_4 . The Kota bunt collections used in 1930 and 1931 consisted of *Tilletia levis* Kühn. The Red Bobs bunt used in 1930 was *T. tritici* (Bjerk.) Wint. with a small percentage admixture of *T. levis*. This chlamydospore collection was the same as collection No. 1 used by Aamodt (1). In 1931 the Red Bobs bunt saved from the parental checks was accidentally destroyed. A composite inoculum from bunted heads of the Red Bobs \times Kota F_3 hybrids was used as Red Bobs bunt in the tests of that year. Microscopic examination showed this collection to consist of approximately one-third *T. levis* and two-thirds *T. tritici*.

Loose Smut Inoculum

The inoculum for the study of reaction toward loose smut, caused by *Ustilago tritici* (Pers.) Jens., was obtained in 1929 from the newly emerged sporulating heads rogued from special Kota and Ceres plots, and from Kota parental check rows in breeding plots. In 1930 inoculum was obtained from similar sources and also from F_3 hybrid plants.

Methods

General

The seed from individual F_2 and F_3 plants (F_3 and F_4 lines respectively) was spaced in five-foot rows one foot apart and handled on the individual-plant basis. Parental checks were grown at intervals of 30 rows with each generation and treated in the same manner as the hybrid material.

The F_3 was grown in three series. The first series was grown in a genetic and loose smut nursery. The second was in a bunt nursery where the seed was inoculated with chlamydospores from Kota, and the third, when there was sufficient seed, in a bunt nursery where the seed was inoculated with chlamydospores from Red Bobs.

Bunt Inoculation

The bunted heads from the sources already described were passed through a small hand grinder that reduced the whole to a fine powder. Care was taken to avoid contamination of each lot by spores from the other. The inoculum was applied to the seed by shaking well a small portion of the ground chlamydospores in the envelope with the seed. The quantity used was approximately 1 part of inoculum to 25 of seed by weight.

In the taking of bunt data, counts were made in the field at maturity. Each head was carefully examined, and a plant was considered to be infected if one or more florets on any head proved to be bunted.

Only those F_3 lines showing a combination of earliness, good strength of straw, and a degree of bunt resistance similar to Garnet were tested in F_4 for reaction to Kota bunt and Red Bobs bunt. These F_4 tests were made

on the progeny of six plants from each selected line grown in short 25-seed rows, and a further test made on a 50-seed row inoculated with a composite of the two bunt collections.

Loose Smut Inoculation

The F_2 plants were inoculated with loose smut by brushing the wheat heads with a small brush of 10 to 20 infected spikes. This was done three times at three-day intervals during the flowering period of the hybrids and parental check plants. When each treatment was complete the spikes were covered with a brown smudge of spores. The weather during this period was clear and dry.

A minimum of five heads from as many plants in each F_3 line of population No. 109 of the Kota \times Garnet cross were inoculated by Stringfield's method (48). Spaced plant-rows of 20 to 30 seeds each of this material were grown in 1931 to check in F_4 the F_3 findings. In a preliminary test in the greenhouse this inoculation method yielded as high as 75% infection.

Bunt

Considerable literature dealing with breeding and inheritance studies on wheat in relation to bunt reaction has been well reviewed by Gaines (13), Briggs (3, 4) and more recently by Aamodt (1). Consequently mention need only be made in this paper of those authors whose works have a direct bearing on the results reported herein.

The population of many of the F_3 lines tested for bunt reaction was reduced to a few plants by cutworms and a prolonged period of soil drifting in the spring of 1930. This was particularly true of lines located at the western and northern limits of the bunt nursery. However, since the distribution of the lines was essentially on a random basis in the seeding plan, a reduction in the amount of data from which to draw conclusions was the only appreciable effect of this damage. In summarizing the data, all lines containing less than ten plants were disregarded, the amount of infection was expressed on a percentage basis, and the lines were grouped in classes according to the percentage of bunted plants. A class interval of 10% was used, the bunt classes being described as 5, 15, 25, etc., denoting the infections within the ranges from 1 to 9%, 10 to 19%, 20 to 29%, etc., respectively. A zero class was added to include all lines free from bunt.

In addition, the percentage infection of the entire parental population used as checks was computed and is shown in Table I. This seemed advisable since only two rows of the Red Bobs checks in the Red Bobs bunt series contained the minimum number of ten plants required for the method of summarization just described.

The lower percentage of infection obtained in 1930 in all three varieties inoculated with Red Bobs bunt might be interpreted as due to a weakness in the infection capacity of that particular lot of bunt, lack of maturity, or artificial injury to the sample, rather than to its inherent infection capabilities. However, in view of the difference in behavior of the two lots of bunt on

Kota and Red Bobs, it must be concluded that the original collections consisted essentially of differing physiologic forms or groups of forms. The virulence of the original Red Bobs bunt appeared to be considerably less than that of the Kota bunt. The reaction of the three varieties to Kota bunt was substantially the same in both years. This cannot be said of the Red Bobs collections where a like reaction was hardly to be expected in view of the different sources of origin and the differences in species composition as revealed by the microscope.

TABLE I
AVERAGE PER CENT SMUT-INFECTED PLANTS OF PARENTAL VARIETIES

Smut	Variety					
	Kota		Red Bobs		Garnet	
	1930	1931	1930	1931	1930	1931
Kota bunt	68.3	54.8	51.2	51.8	10.4	8.1
Red Bobs bunt	30.0	68.4	36.0	51.6	2.6	9.7
Loose smut	1.5	20.4	0.0	—	0.6	5.1

At the beginning of this study the two populations in each cross were considered separately. The results for the two populations proved to be so similar that it was decided to bulk the data. This decision was based, in part, on the data presented in Table II. These data may be taken to indicate not only the identity of the duplicate populations but also the uniformity of the conditions of test, and they bear out the assumption made in regard to the randomness of sampling in the material included for study.

TABLE II
DISTRIBUTION INTO INFECTION CLASSES OF F_2 LINES OF KOTA \times RED BOBS AND KOTA \times RED BOBS AND KOTA \times GARNET HYBRID POPULATIONS, WHEN INOCULATED WITH TWO COLLECTIONS OF BUNT

Cross	Kind of inoculum	Population No.	Numbers in percentage infection classes												
			0	5	15	25	35	45	55	65	75	85	95	Total	
Kota X Red Bobs	Kota bunt	115				1	3	15	28	34	32	20	10	143	
		118				5	15	22	26	33	34	8	2	145	
Kota X Red Bobs	Red Bobs bunt	115		1	7	13	11	16	7	9	3	1		68	
		118	2	7	10	11	23	17	9	7	3			89	
Kota X Garnet	Kota bunt	109	2	6	7	13	28	27	12	19	5	4	1	124	
		112	1	6	11	22	29	22	13	10	8	2	1	125	
Kota X Garnet	Red Bobs bunt	109	57	32	20	7	3	1		1				121	
		112	30	35	23	9	9	5	2					113	

A study was made of the distribution in the infection classes of the lines that had ten or more plants in both bunt tests. As this reduced number of strictly comparable lines followed very closely in distribution the total population in each cross it was decided to use the larger numbers.

The data in Table III show very well the heritable nature of bunt reaction. In the susceptible \times susceptible crosses (Kota \times Red Bobs) the hybrids occupy the entire parental infection range and no segregates more resistant than either parent appear. The majority of lines fall in the classes showing 50 to 80% infection. These results are in agreement with those obtained by Briggs (3) in Baart \times Hard Federation crosses.

TABLE III

DISTRIBUTION INTO INFECTION CLASSES OF PARENTAL VARIETY ROWS AND F_2 LINES WHEN INOCULATED WITH BUNT FROM KOTA (SERIES 2)

Parents and crosses	Numbers in percentage infection classes											Total of lines
	0	5	15	25	35	45	55	65	75	85	95	
Kota	—	—	—	—	1	4	—	5	4	4	2	20
Red Bobs	—	—	—	2	—	2	3	2	—	—	—	9
Garnet	2	2	5	—	—	—	—	—	—	—	—	9
Kota \times Red Bobs	—	—	—	6	18	37	54	67	66	28	12	288
Kota \times Garnet	3	12	18	35	57	49	25	29	13	6	2	234

The Kota \times Garnet segregation shows distinctly the introduction into the hybrids of factors resistant to Kota bunt. In discussing hybrids of which Garnet is a parent it must be remembered that the resistance of this variety is much greater in degree than the number of infected plants would indicate. Bunted Garnet plants seldom showed more than one partly bunted head, whereas bunted Kota and Red Bobs plants usually showed several. In some cases every culm of these varieties bore a bunted head. A considerable number of the hybrids showed resistance equal to that of the Garnet parent, a larger number exhibited the high susceptibility of the Kota parent, but the majority fell in the classes showing 20 to 50% of the plants bunted.

TABLE IV

DISTRIBUTION INTO INFECTION CLASSES OF PARENTAL VARIETY ROWS AND F_2 LINES WHEN INOCULATED WITH BUNT FROM RED BOBS (SERIES 3)

Parents and crosses	Numbers in percentage infection classes											Total
	0	5	15	25	35	45	55	65	75	85	95	
Kota	—	—	6	7	2	2	3	1	—	—	—	21
Red Bobs	—	—	1	—	—	—	1	—	—	—	—	2
Garnet	6	3	—	—	—	—	—	—	—	—	—	9
Kota \times Red Bobs	2	8	17	24	34	33	16	16	6	1	—	157
Kota \times Garnet	87	67	43	16	12	6	2	1	—	—	—	234

The data in Table IV show that the behavior of the Kota \times Red Bobs hybrids toward Red Bobs bunt resembles their reaction toward Kota bunt, except that the whole distribution is moved toward the lower infection classes. The Kota \times Garnet hybrids show a considerable aggregation in the non-bunted class and the two lowest infection classes.

Loose Smut

There is a dearth of literature on inheritance of resistance to loose smut of wheat, probably because no rapid and effective method of inoculating the wheat flower has been devised. The laborious method of opening and inoculating each flower by hand during the short period when infection can take place probably has been the factor limiting such studies.

Olson, *et al.*, (40) in 1920, were the first to report on inheritance of resistance to this smut. A review of their work by Matsuura (33) states, "there are several genes involved for resistance to smut caused by *Ustilago tritici* in different wheat varieties". These are said to be transmitted independently and to have a cumulative effect when they act together. No mention is made of the methods used in arriving at these conclusions.

Tisdale and Tapke (51) pointed out in 1927, as a result of inoculation studies, that highly resistant and immune strains were found in many varieties of common wheat. Fultz, Fulcaster, Hussar, and Ridit, winter varieties, and Preston, a hard red spring wheat, each contained such strains.

Later extensive tests by Tapke (49) supplemented this list of varieties and confirmed the observations on field resistance in certain varieties by showing these same varieties to be resistant following artificial hand-inoculation. On the whole the club wheats tested were found to be very susceptible and Kota, a common wheat, showed the same degree of susceptibility. The use of composite samples of spores, instead of spores from single heads, in the latter years of this test showed that some varieties, thought to be resistant or immune, were highly susceptible. The presence of physiologic forms of the pathogene was demonstrated by this change in the reaction of certain varieties.

In a test of six years duration on the continued selection of large and small seed, an increase in amount of loose smut in the plots sown to small seeds was noted by Taylor (50). He suggested that this was probably due to some effect of the loose smut organism within the developing kernel on endosperm development and not to any heritable factors.

In a study which demonstrated that *Ustilago tritici* possessed physiologic strains Piekenbrock (43) made resistance and susceptibility tests on many European varieties and hybrids. Later, crosses were made between a highly resistant variety, Grüne Dame, and two susceptible wheats, Rumker's Dickkopf and Rimpau's Schlanstedter. Segregation was such as to indicate that immunity was inherited as a recessive. This author describes an inoculation mechanism and technique that would appear to be an improvement on the usual methods.

Grevel (17), another German worker, confirmed Piekenbrock's conclusions that resistance to loose smut in wheat is inherited as a Mendelian recessive. Neither of these workers, however, was able to formulate a simple factorial explanation. Grevel obtained strains from two crosses of resistant \times susceptible varieties that were immune from biotypes I and II of the four physiological strains he has established in *U. tritici*.

Preliminary tests made in the greenhouse during the winter months of 1929-1930 indicated that the percentage of infection obtained from inoculating with loose smut was very low. This was borne out later by the field studies. Only three of the lines showing infection in the genetic series were infected in the two bunt series. In view of this condition it was decided to make no distinction among the three series, but to summarize the results on the basis of the three replicates as one. Only a few lines had a total of less than 25 plants. A line was considered to be infected when one or more heads or parts of heads on any plant produced loose smut. Few lines showed more than one or two infected plants. The data are given in Table V.

TABLE V
LOOSE SMUT INFECTION OF PARENTS AND F_3 HYBRID LINES FROM KOTA \times RED BOBS
AND KOTA \times GARNET CROSSES

Parents and crosses	Total lines	Number of lines infected	Percentage of lines infected
Kota	26	12	46.2
Red Bobs	15	0	0.0
Garnet	12	3	25.0
Kota \times Red Bobs (115)	200	40	20.0
Kota \times Red Bobs (118)	195	42	21.5
Kota \times Garnet (109)	164	69	42.1
Kota \times Garnet (112)	179	73	40.8

The reaction of the hybrids is in agreement with that of the parents in each cross. Forty-six per cent of the Kota checks were infected, 25% of the Garnet checks and 42.1% and 40.8% in two separate populations of the F_3 Kota \times Garnet hybrids. The two Kota \times Red Bobs populations had 20 to 21.5% of the F_3 lines infected, showing an intermediate condition between the 46.2% infection of the Kota parent and the immunity of the Red Bobs parent. The close check between similar populations in the same cross with regard to the percentage of lines showing infection is of interest in view of the low degree of infection obtained, and is further evidence that the two populations are similar for factors governing the reaction to loose smut.

TABLE VI
SUMMARY OF REACTION OF KOTA \times GARNET F_3 AND F_4 LINES
(POPULATION NO. 109) TO *U. Tritici*

F_3 reaction (1930)		F_4 reaction (1931)		
Infection	Number of lines	No infection	Some rows infected	All rows infected
None	53	3	38	12
Some	37	4	23	10
Totals	90	7	61	22

One hundred and sixty-four F_4 lines were sown in 1931. Owing to heavy rains in midsummer 74 of these were drowned out. Of the 90 remaining, 53 lines which had shown no infection in 1930 gave the following reaction in 1931: 3 continued to show no infection, in 38 some rows showed infection and in 12 all rows were infected. The infected rows in the last two classes had an average infection rate of 12 and 24% respectively. Of the 37 lines which had shown some infection in 1930, 4 showed no infection, in 23 some rows were infected, and in 10 all rows were infected. The infection percentages in infected rows in the last two classes were 12 and 20% respectively. These data are summarized in Table VI and demonstrate that there is not sufficient correlation between the infection in the two generations to make a genetic analysis. This may be due in part to the inadequacy of the 1930 tests, as a result of faulty inoculation technique, or to the possible presence of physiologic forms of the pathogene in the inoculum.

Consideration of the data presented in Table I would appear to indicate that a certain amount of resistance to loose smut must be present in Garnet. Of the 17 check rows of Kota 14 showed infection, which on a plant basis gave an infection rate of 20.4%. On the other hand only 9 out of the 22 Garnet check rows showed infection and the infection rate on a plant basis was 5.1%. It may be that both parents are similar in physiological susceptibility to the inoculum used but there may exist some functional difference in period of glume opening, in degree of glume opening or in the length of time glumes remain open during the flowering period. Any of these factors would vary the opportunity of obtaining infection under the conditions of test. Such phenomena would presumably be characteristic in a given variety and would explain the constant reaction of the parental varieties as well as the perfectly random manner in which the hybrids were affected in these tests.

Seed Color

Since seed color is a striking characteristic of any wheat variety, and of considerable economic importance, it is not surprising that it was one of the first characters genetically investigated in the wheat species. Biffen (2) was the first to report on the inheritance of red kernel color in wheat. He found red to be dominant over white in F_1 and to segregate on a single factor basis in F_2 .

The studies of Nilsson-Ehle (36, 37, 38, 39) on color inheritance in wheat established both two- and three-factor hypotheses and laid the foundation for the present conception of the inheritance of quantitative characters. An analysis of his F_2 results led him to postulate that the red grain color of the "Sammet" parent was due to the presence of three dominant and independent factors, R_1 , R_2 and R_3 , each of which by itself was capable of producing redness. He explained the varying shades of red obtained as due to "additive or cumulative action of the several color factors", the presence of two or three factors giving twice or thrice the depth of color that one factor would produce. Only when the three factor pairs were homozygous for red would

the original dark red of the red parent be recovered. The whites would all breed true since they must be homozygous recessive, and between these extremes would be five shades of red. Intermediate types of red could not be readily separated on the basis of color intensity alone and recourse was made to classification on the basis of breeding behavior. Nilsson-Ehle was the first to record the occurrence of white grained plants in the F_2 from a red \times red cross (39).

Howard and Howard (28, 29), working about the same time as Nilsson-Ehle, published in 1912 and 1915 evidence of a similar nature showing the existence of two factors for red in some Indian varieties of *T. vulgare*, and the presence of three factors for red in a *T. compactum* known as "American Club".

In a series of crosses in Washington, 1917, between several club varieties and several common wheats, Gaines (12) confirmed the two- and three-factor hypothesis of Nilsson-Ehle. In analyzing his results he used only three red color classes. He assumes that each factor for R produces the same color of red whether heterozygous or homozygous, and that the effect of the different factors R, R', and R'' is cumulative. These assumptions differ but little from those of Nilsson-Ehle, the differences being due to the difficulty in distinguishing between the shades of color.

On the whole, Gaines' results support the assumption of three independent factors working together to produce the red and white color phenomena in wheats. He points out, however, that it is not sufficient to explain all the color inheritance results found. He reports two cases in which red seed was produced from a white \times white cross. This would indicate that another factor or set of factors was concerned, which would probably be inhibitory in effect.

Percival (42) states that the usual result of crossing two white-grained wheats is the production in subsequent generations of white-grained plants only. He reports, however, that two workers found otherwise. Pitsch and Vilmorin obtained red-grained plants in different crosses in which both parents were white-grained. These results, with a few discrepancies to be noted in some of Nilsson-Ehle's work, lend support to the possibility of some inhibitory factor or set of factors operating in color inheritance.

Since these earlier investigations many others have been undertaken. Harrington (19) studied some of our common wheats and concluded that Kitchener contained two independently inherited factors for red grain color. In some strains of Red Bobs and Hard Red Calcutta he found two factors, in others one. This probably indicates that these two varieties are made up of red strains that are indistinguishable, one of which has a single factor pair while the other has two pairs for red. Awning and seed color were studied in these crosses and were found to be independently inherited.

Harrington and Aamodt (22) found no relation between seed color and resistance to *Puccinia graminis tritici* but report a 3 : 1 ratio for red and white color in the F_2 of the durum crosses they studied.

Clark (6) reports on several reciprocal crosses between Hard Federation and Kota. He obtained a fairly good agreement with a theoretical two-factor ratio. When Kota was used as the female parent a much better fit was obtained than when the reciprocal cross was made, suggesting a possible slight maternal influence.

Hayes and Robertson (27) worked with red \times white crosses using Bobs as the white parent. The Marquis \times Bobs crosses, made reciprocally, gave a 15 : 1 ratio indicating that Marquis carries two independent factors for red. The red \times red crosses gave a 63 : 1 ratio for red and white in F_2 . The results were explained on the basis of two factors for red in Marquis and a single but different factor in Minturki and Kanred, the other varieties used. In one cross the "Marquis" parent was proved to be Red Fife, and, since no disturbance of the expected two-factor ratio occurred, it may be surmised that Red Fife has a complement of color factors similar to that of Marquis.

Clark and Hooker (9), like all former workers, found red to be dominant over white. They used reciprocal crosses of Marquis and Hard Federation, and in 10 families out of 12 studied, obtained a very good fit for a 15 : 1 ratio, indicating that two factors are concerned with red kernel color in Marquis. The two other families gave a satisfactory fit for a 3 : 1 ratio indicating that a single factor only was concerned. This difference in the ratios obtained indicates, according to Clark and Hooker, that either Marquis does not have homozygous dominant factors for red-kernel color, or the Hard Federation parents differed in their genotypes.

Stewart (45) has recently obtained segregation on a three-factor basis in a pure line red \times white cross, using Kanred as the red parent. Correction of the F_2 analysis was made in F_3 and, although an excess of true-breeding reds and a deficiency in the segregating classes was found, this was explained by the fact that only 30 to 40 plants in each progeny were available. Such numbers were not thought to be sufficient to show the one plant with white grains where a 63 : 1 segregation was expected. Stewart's findings are not in accord with those of Hayes and Robertson (27) who found only one factor for red in Kanred.

Stewart and Tingey (47) found duplicate factors for red color in the cross Marquis \times Federation (red \times white). An attempt was made to correlate glume and seed color factors but these proved to be inherited independently. When a light-red kernelled variety, Odessa, was used as one parent in a red \times white cross, Stewart and Price (46) found a single factor difference responsible for color segregation.

Matsuura (33) reports that several European investigators working with various species and varieties also found mono-, di- and trigenic ratios in color inheritance.

In the work reported here, only the Kota \times Red Bobs crosses were studied for seed color, since no segregation for this character took place in the Kota \times Garnet crosses. The seed from the F_2 plants was classified as either red

or white. In F_2 twenty-five plants were taken on which to determine color segregation in each line. Since there were seldom twenty-five plants available in the genetic series rows, the deficiency was supplied by plants taken at random from the bunt-free population in replicates of the Kota bunt and Red Bobs bunt series. Seed color determinations were made as in F_2 by ocular examination.

The segregation for seed color noted in the Kota \times Red Bobs F_2 hybrids clearly indicated that a two-factor difference for color exists in this cross. Table VII shows the F_2 results. The odds of 1.31 : 1 indicate a very good fit.

TABLE VII
SEGREGATION IN F_2 FOR SEED COLOR IN KOTA \times RED BOBS CROSSES

Color class	Number		Dev.	P.E.*	$\frac{D.}{P.E.}$	Odds
	Observed	Calculated				
Red	376	372.2	3.8	3.25	1.16	1.31 : 1
White	21	24.8				

* Probable errors for numbers of individuals given here and elsewhere in this paper were obtained from tables of probable errors of Mendelian ratios, prepared by the Department of Plant Breeding, Cornell University, Ithaca, N.Y.

A population segregating on a 15 red : 1 white basis in F_2 , is expected to give a 7 : 4 : 4 : 1 ratio in F_3 . That this expectation was not fulfilled in this case is shown by the data in Table VIII.

TABLE VIII
SEGREGATION IN F_3 FOR SEED COLOR IN KOTA \times RED BOBS HYBRIDS

Color class	Expected ratio	Number		O-C	(O-C) ²	$\frac{(O-C)^2}{C}$
		Observed	Calculated			
Red	7	324	168.9	155.1	24056.0	142.43
15R : 1W	4	15	96.5	81.5	6642.3	68.83
3R : 1W	4	26	96.5	70.5	4970.3	51.51
White	1	21	24.1	3.1	9.6	0.40

P = extremely small

$X^2 = 263.17$

The fit for the segregating classes and for the true-breeding red class is very poor. The discrepancy may be due in part to the difficulty in distinguishing, in vitreous grain samples, between very pale red types and those having no pigmentation; or to the classifying of some white samples as pale red as a result of their discoloration by disease. The most probable cause, however, may be the small numbers used to test segregation in F_3 . In most cases 25 plants in each line were used for this purpose. It is believed that

many lines assigned to the apparently true-breeding red class would have shown segregation if larger numbers had been employed. This explanation was advanced by Stewart (45) to explain a similar deficiency in his segregating classes.

Since a two-factor difference for color is indicated in this red \times red cross it would appear that Red Bobs and Kota each carry one factor pair for red which is unlike that carried by the other. Harrington (19) found two factors for color in some strains of Red Bobs and one factor in others. Clark and Hooker (9) have reported the same condition to hold for Marquis. An even more striking contrast exists in the finding of one factor for red by Hayes and Robertson (27) and three factors by Stewart (45) in the variety Kanred. The findings here reported are at variance with those of Clark (6) who found seed color in Kota to be governed by two factors. On the whole it would appear that many commonly grown varieties of wheat are not homogeneous for color. Lack of homogeneity within these varieties doubtless exists in relation to other characters and its demonstration serves to emphasize the presence and probable importance of strains and pure lines in the common varieties.

Awns

The literature on inheritance of awns is quite voluminous and much confusion appears in the early studies due to inexact and faulty classification of awn types. All lines not fully awned were usually considered as awnless and nearly all results were interpreted on the basis of a single partially dominant factor for awns. This early work is well summarized by Percival (42), Kezer and Boyack (30), and Gaines (12). They reported a single factor difference for awning in the varieties studied but disagree in regard to the partial dominance of awning or awnlessness. The Howards (28, 29) were the first to recognize differences in the so-called awnless groups. They established the presence of two factors controlling the length of tip awns within that class. Harrington (19) used their method of awn classification and from appropriate crosses obtained both single factor and two factor results.

At present two systems of awn classification are in common use. One, essentially the same as that used by the Howards, is now used by most American investigators. A description of the five awn classes employed in this system given by Clark (6), follows:—

“(1) awnless, (2) apically-awnletted, (3) awnletted, (4) short-awned, and (5) awned. Class 1, awnless, normally is entirely without awnlets in the apical part of the spike, although a few awnlets 1 to 2 mm. long may occur at the apex under abnormal conditions. Class 2, apically-awnletted, has awnlets 2 to 20 mm. long at the apex of the spike but rarely extending to the central and basal portions. Class 3, awnletted, has awnlets from 3 to 40 mm. long, the shorter occurring at the base of the spike and the length increasing toward the apex. Class 4, short-awned, has short awns throughout, varying from 15 to 50 mm. long but only about half the length of the normal awns. In Class 5, awned, the awns vary from 30 to 80 mm. in length.”

Clark found that these classes were fairly definite and that any doubt regarding classification would be cleared up by studying the breeding behavior in F_3 . The other system which involves only four true-breeding awn classes, was described by Stewart (44). Although considerable difficulty is said to be encountered in classifying intermediate types in F_2 a study of the F_3 breeding behavior makes for accuracy and the method is considered adequate by Stewart and coworkers (45, 46, 47). A description of the classes follows:—

"Class 1, awnless, or almost so; class 2, short-tip awns; class 3, short-tip awns in lower half of spike and part-length awns in upper half; and class 4, fully awned."

The detailed findings of the various investigators in regard to the numerous crosses studied for the inheritance of awning will not be recounted here. However, a few of the results obtained from linkage studies may be mentioned briefly. The importance of a possible linkage between awn factors and factors governing resistance or susceptibility to disease is obvious. So also is any association that may be found between awns and yield, quality of grain, or other agronomic quality. Although numerous tests for linkage have been made in awn studies to date no linkage has been reported between awn factors and factors for either rust or smut reaction (14, 16, 21, 34). In regard to yield and protein content some indications of relation exist. Hayes (24), after studying crosses of Marquis on Preston and Bluestem, concluded that "the bearded families average somewhat higher than the awnless families for average length of seed, average percentage of plumpness of seed, and average yield per plant". Later, he and coworkers (26) found that the awned lines of spring and winter wheats, on the average, excelled in plumpness of grain and that a high degree of correlation existed between plumpness of grain and yield. Clark and coworkers in a series of studies (6, 7, 8, 11) found varying degrees of direct relation between length of awn and crude protein content, and noted in all except one case a positive correlation between awn length and yield. In the exceptional case (11) opposite results were obtained.

The two crosses described in foregoing sections were studied in F_2 and F_3 for segregation of awn characters. The five awn classes of Clark (6), already described in the literature review, were used as a basis of classification in this study.

In F_2 an attempt was made to place each plant in one of the five classes. In addition, when the plant did not appear to fit the centre of the class range, an estimate was recorded by the use of plus and minus signs to indicate whether the plant approached the upper or lower limit of the class. Also an average measurement, taken in millimetres, was recorded for the awn and beak. These measurements were taken near the centre of the awn or awnlet range and consisted of four readings on from two to four well-developed heads.

The F_3 lines were classified plant by plant, no attempt being made to make any finer classification than that provided by Clark's five awn classes. At this stage the three replicates were checked against each other to eliminate

any errors that might have crept in during the handling and preparation of the seed or in seeding. Thus a better judgment of the purity of the individual lines was obtained and data for material damaged while in the stook were readily corrected. Except in cases where such damage occurred, awn classification was made on the material grown in the genetic series.

The F_2 segregation for awns in the Kota \times Red Bobs crosses agreed very closely with the findings of Clark (6) in some Kota \times Hard Federation crosses. His grouping of the awnless classes 1, 2, and 3, and the awned classes 4 and 5, gave a 3 : 1 ratio with odds of 1.46 : 1, indicating a close fit. A similar grouping of the segregating F_2 Kota \times Red Bobs hybrids is shown in Table IX. The F_2 classification has been corrected by the breeding behavior of F_3 in these data. The odds of 2.46 : 1 against the occurrence, due entirely to chance, of a deviation as great or greater than the one observed, indicates a good fit to the theoretical ratio.

TABLE IX

SEGREGATION FOR AWNING IN KOTA \times RED BOBS CROSSES IN F_2 , AND CALCULATION OF GOODNESS OF FIT TO A 3 : 1 RATIO

Awn class	Numbers		Dev.	P.E.	D. P.E.	Odds
	Observed	Calculated				
1	49	297	9	5.72	1.57	2.46 : 1
2	108					
3	140					
4	14	87				
5	73					

Awn class 1—awnless; Awn class 2—apically-awnletted; Awn class 3—awnletted; Awn class 4—short awned; Awn class 5—awned.

While the presence of one major factor difference is indicated by these and by Clark's results, it is probably concerned principally with the presence or absence of the long, strong Kota awn. The presence of true-breeding lines in classes 2 and 3 requires a two- or multiple-factor hypothesis. Unless a detailed analysis were made of the breeding behavior of the F_3 segregates in F_4 it would be impossible to say more than that in these crosses, two or more factors for awns must be involved, and that the segregation already obtained resembles the results of Clark and others (6, 8) in crosses of a similar nature.

In the Garnet \times Kota hybrids the segregating F_2 population could be grouped, on the basis of F_3 breeding behavior, into three classes. These were awn classes 3 and 5, and a segregating class. Although several F_2 plants had been placed in class 4, all of these were observed to segregate in F_3 . Ten plants, classified as class 2 in F_2 , were included in class 3 as were the few lines segregating for classes 2 and 3. The findings of other workers

TABLE X

SEGREGATION FOR AWNING IN KOTA \times GARNET HYBRIDS IN F_2 , AND CALCULATION OF GOODNESS OF FIT TO A 1 : 2 : 1 RATIO

Awn class	Numbers		O - C	(O - C) ²	$\frac{(O - C)^2}{C}$
	Observed	Calculated			
3	58	85.7	27.7	770.1	9.88
Segregating	173	171.5	1.5	2.3	0.01
5	112	85.7	26.3	689.1	8.04

P = 0.00013

 $\chi^2 = 17.93$

on similar crosses have shown that a one factor difference explains most of the results satisfactorily. However, the data in Table X show clearly that a single factor difference does not very well explain segregation for awns following a cross of Garnet and Kota. The method of classification used was not, in the opinion of the author, entirely satisfactory. The deficiency in class 3 and the excess in class 5 cannot be explained on the basis of errors in classification. It must consequently be concluded that probably more than one factor is involved in awn segregation in Kota \times Garnet crosses.

Strength of Straw

The ability to resist lodging and stand erect is a very important agronomic character in a variety of wheat. Straw that will resist lodging when conditions are unfavorable is sought in all hybrid selection work which aims at the improvement of the present varieties of wheat. However, strength of straw is a character that varies so greatly with differences in environment that few genetic studies have been made on it and no factorial explanation of its inheritance attempted.

The Howards (28) made a study of the standing power of wheats which they believed to be due to strong straw and the power to form a strong root system. They made crosses between a *vulgate* form with strong straw but inferior rooting capacity and another variety with weak straw but good rooting power. In F_2 all combinations of these characters appeared. This would seem to indicate independent inheritance of the two characters considered by the Howards to produce good standing power.

Harrington (20) made a study of erectness of plant in the parents and hybrids of two durum crosses. Grown in two series of rows the F_4 families of Mindum \times Pentad gave a correlation of $r + 0.389 \pm 0.037$ and of Kubanka No. 8 \times Pentad a correlation of $+ 0.537 \pm 0.041$. Transgressive segregation occurred in both crosses. He concluded "that in both crosses erectness is dependent on several heritable factors, part of which are present in one parent and part in the other". A slight linkage between factors governing rust resistance under field conditions and those governing erectness of plant was noted.

Waldron (52) points out that an attempt was made to secure a strong-strawed rust-resistant line from Kota \times Marquis crosses. Marquis has a strong straw while Kota is rather weak. Differences in strength of straw were very apparent in the selections made, the range being from about the strength of Kota to midway between the two parents. No rust-resistant line with strength of straw equal to Marquis was found. However, this author believes the occurrence of such a form to be possible. One resistant selection showed considerably weaker straw than the Kota parent.

An association of awnlessness and increase in lodging in Propo hybrids grown at Davis, California, was noted by Clark, *et al.* (8). Goulden and Neatby (15) have reported that a genetic linkage appears to exist between weakness of straw and mature plant resistance to rust. This linkage, it is pointed out, may be broken if a large enough hybrid population is grown to permit the detection of the few cross-over types.

An attempt was made to classify the strength of straw or standing power in each row of the three F_2 series for all four populations. The parental check rows were first studied and the standing power of the parents taken as the basis of classification. For the purposes in view, upstanding rows of Red Bobs and Garnet were considered as strong and designated as "S". In each of these varieties some rows, affected by the fluctuations of environment, appeared less upstanding than the strong rows. These were classed as midstrong and designated as "MS". The midrange of the Kota straw strength was taken as representative of the weak, or "W" class. Kota showed a considerable tendency toward the production of knee-bent culms that spread outward from the upright plane of the row, and all culms showed a tendency to criss-cross within the row or to fall into or against adjacent rows. An intermediate class was made between the midstrong class and the weak, into which many of the stronger-appearing Kota parent lines fell, and a weaker class was set up to include those lines which showed distinct signs of lodging. These were known as midweak, and very weak, and designated "MW" and "VW" respectively.

Determinations were made following a period of wind and rain. At this time the earlier lines were in the dough stage but still green, and the later lines contained large watery kernels. About one week later these determinations were checked. No attempt was made to distinguish heterozygosity, the standing power of the whole row being taken into consideration. A final rating was made, where the rating in the replicates was at variance, by (1) taking the midclass if three contiguous ratings had been made, (2) by taking the majority rating where two were in agreement, and (3) by making adjustments in favor of the class rating represented by the largest number of plants where the lines were badly reduced in number by the epidemic of cutworms and the wind damage already referred to.

Examination of the data in Table XI reveals a complex condition. A leaning toward greater weakness than that found in Kota is evident in both crosses. If transgressive segregation were not taking place the expected

TABLE XI

DISTRIBUTION FOR STRENGTH OF STRAW IN PARENTS AND F_2 HYBRID LINES FROM
KOTA \times RED BOBS AND KOTA \times GARNET CROSSES

Parents and crosses	Numbers in classes for straw strength					Totals
	S	MS	MW	W	VW	
Kota	—	3	50	21	3	77
Red Bobs	33	11	—	—	—	44
Garnet	19	15	2	—	—	36
Kota \times Red Bobs	15	64	114	148	52	393
Kota \times Garnet	34	74	78	120	35	338

S = Strong; *MS* = Midstrong; *MW* = Midweak; *W* = Weak; *VW* = Very weak.

numbers of hybrid lines in the "VW" class would be of the order of 11 and 8 instead of 52 and 35 respectively.

The data were fitted to a theoretical single factor ratio. To do this the "S" and "MS" classes were grouped as "Strong" while the "MW" and "W", and "VW" classes were grouped as "Weak". Odds against the occurrence, due to chance, of deviations as great or greater than the ones obtained were 39 : 1 and 216 : 1 in the Kota \times Red Bobs and Kota \times Garnet crosses respectively. This indicates that the segregation cannot be explained on a single-factor basis.

In attempting to fit the data to a two-factor ratio it was assumed that the factors for strength of straw, designated *S* and *S'*, were cumulative in effect and partially dominant. Since no attempt had been made to distinguish the heterozygous from the homozygous lines when taking the data it was expected that the classes set up on the basis of phenotype would contain progeny of plants differing in genotype in F_2 . The grouped weak and very weak classes would be derived from the *sss's'*, *ssS's'*, *Sss's'*, *SSs's'* and *ssS'S'* genotypes, the midweak class from the *SsS's'* genotype, the midstrong class from the *SSS's'* and *SsS'S'* genotypes and the strong class from the homozygous dominant, *SSS'S'*. The four classes so derived would be in the ratio of 7 : 4 : 4 : 1 respectively. Calculations showing the goodness of fit to this

TABLE XII

STRENGTH OF STRAW SEGREGATION IN KOTA \times RED BOBS F_2 HYBRID LINES AND
CALCULATIONS OF GOODNESS OF FIT TO A TWO-FACTOR RATIO

Strength class	Expected ratio	Numbers		$O-C$	$(O-C)^2$	$\frac{(O-C)^2}{C}$
		Observed	Calculated			
VW + W	7	200	171.9	28.1	789.6	4.59
MW	4	114	98.3	15.7	246.5	2.51
MS	4	64	98.3	34.3	1176.5	11.97
S	1	15	24.6	9.6	92.2	3.75

$$P = 0.000045$$

$$X^2 = 22.82$$

TABLE XIII

STRENGTH OF STRAW SEGREGATION IN KOTA X GARNET F_2 HYBRID LINES AND
CALCULATION OF GOODNESS TO FIT TO A TWO-FACTOR RATIO

Strength class	Expected ratio	Numbers		O - C	(O - C) ²	$\frac{(O - C)^2}{C}$
		Observed	Calculated			
VW + W	7	155	149.2	5.8	33.6	0.23
MW	4	78	85.3	7.3	53.3	0.62
MS	4	74	85.3	11.3	127.7	1.50
S	1	34	21.3	12.7	161.3	7.57

$$P = 0.0194$$

$$X^2 = 9.92$$

phenotypic ratio are given in Tables XII and XIII. In this case also the expected ratio was not close enough to the obtained results to warrant the conclusion that segregation for this character was entirely on a two-factor basis.

From a consideration of these attempts to fit the segregation for straw strength to single factor and two-factor ratios it would appear that proof for either explanation could not be offered. But if the difficulties of classification are taken into consideration a two-factor difference might well be indicated by the Kota X Garnet data. No attempt was made to test for goodness of fit to a three-factor ratio, since it was believed that the classification was not fine enough; nor could it be made with sufficient accuracy on a character that is so easily modified by environment. It was concluded that the complex segregation noted in this study of strength of straw could be explained best on a multiple factor basis, the presence of two main factors being indicated.

Correlation Studies

The importance of linkage studies in plant breeding cannot be too strongly stressed. In connection with studies on the inheritance of disease reaction the use of correlation as a tool to uncover linkage relations is doubly important. A specific test must, as a rule, be made to determine the presence of factors for resistance and susceptibility. This would be less necessary if such factors were linked with some easily distinguishable morphological character. Under varying environmental conditions it is not always possible to obtain an adequate disease reaction test. Thus the process of selecting desirable, disease resistant agronomic forms from a hybrid population is made difficult. Linkage relations when found are seldom of a complete nature, i.e., a certain amount of crossing-over occurs. A knowledge of the strength of linkage is therefore important in estimating the chances of obtaining a suitable recombination where linked desirable and undesirable characters enter a cross.

That qualitative characters may be linked with quantitative and other characters has been amply demonstrated. Lindstrom (32) found that the number of rows in the ear of corn (*Zea mays*), a quantitative character, was

associated in inheritance with cob, aleurone, and endosperm color as well as with endosperm texture, all of which are simple qualitative characters. Griffiee (18), working with barley (*Hordeum* spp.) demonstrated very striking linkage relations between morphological characters and reaction to *Helminthosporium sativum*. Three factors were found to be concerned with the production of resistance of a certain type. One of these factors was found to be linked with the factor for two-rowed, one with the factor for rough awn, and another with the factor for white glumes. He reports the linkage of the factor for early heading, a physiological character, with the factor for six-rowed and the factor for susceptibility to *Helminthosporium sativum*.

In the studies here reported an investigation was made of the relation between the reaction of Kota bunt and Red Bobs bunt, and between bunt reaction and some plant characters in the F_2 of Kota \times Red Bobs and Kota \times Garnet crosses. Since the data on bunt reaction were taken on a uniform class basis it was possible to use the correlation coefficient (Pearson's " r ") to measure the degree of correlation between the reactions of the two bunts. In the case of the plant characters the classes used could not be considered to have any specific numerical relation. This necessitated the use of the correlation ratio, η . A comparison of the values of r and η in some distributions by means of Blakesman's test for linearity showed that the correlation ratio was the better measure of correlation for those cases. The probable errors were computed by standard formulas. Although the correlation ratio η does not normally carry a plus or minus sign these were added to indicate whether the general trend of correlation was found to be positive or negative (5, 26, 41).

Kota \times Red Bobs hybrids inoculated with Kota bunt. The two characters, awning and strength of straw were correlated with the reaction of the F_2 hybrid plants to bunt.

Negative correlation ratios between reaction to Kota bunt and awning, and Kota bunt and straw strength were obtained. These were -0.262 ± 0.037 and -0.303 ± 0.036 respectively. This would seem to indicate that the Kota plant character, weak straw, is associated with susceptibility to Kota bunt. Some of the genetic factors governing the expression of this character must be linked with a factor for bunt reaction, and therefore located on the same chromosome. Factors for the Kota plant character, awniness, would seem to be linked with factors for resistance to Kota bunt.

Kota \times Red Bobs hybrids inoculated with Red Bobs bunt. The correlation of the same two characters with reaction to Red Bobs bunt in the F_2 lines of these crosses was positive. The correlation ratio for reaction to Red Bobs bunt and awning was $+0.238 \pm 0.052$, and for reaction to Red Bobs bunt and strength of straw, $+0.204 \pm 0.051$. This would seem to indicate that the Red Bobs character of stronger straw was associated with susceptibility to Red Bobs bunt. Some of the genetic factors governing the expression of this character must be linked with some factor for bunt reaction and therefore located on the same chromosome. In the case of the awnless character

of Red Bobs the significant positive correlation indicates a linkage between awnless factors and resistance to the Red Bobs bunt.

Kota × Garnet hybrids inoculated with Kota bunt. The correlation value of -0.203 ± 0.041 between reaction to Kota bunt and strength of straw indicates that the linkage between factors for susceptibility to Kota bunt and for weak straw, noted in the Kota × Red Bobs crosses, has also been carried into this cross.

The negative correlation ratio of -0.235 ± 0.027 obtained for reaction to Kota bunt and awning also parallels the findings in regard to these characters in the Kota × Red Bobs crosses.

Kota × Garnet hybrids inoculated with Red Bobs bunt. Reaction of Red Bobs bunt correlated with strength of straw in these crosses gave a significant correlation ratio of $+0.191 \pm 0.043$, indicating that as strength of straw increases the susceptibility to Red Bobs bunt also increases. This parallels the results for the Kota × Red Bobs crosses and would indicate that some of the same factors for strength of straw are probably present in both Garnet and Red Bobs.

The reaction to Red Bobs bunt and awning show a correlation ratio of $+0.274 \pm 0.041$. That the relation would be of a positive nature is to be expected if the resistance factor of Garnet is linked with factors for short tip awn.

TABLE XIV

SUMMARY OF CORRELATION RATIOS AND CORRELATION COEFFICIENTS OBTAINED IN STUDIES OF CORRELATED INHERITANCE IN F_2 HYBRIDS OF KOTA × RED BOBS AND KOTA × GARNET CROSSES

Characters and reactions correlated	F_2 hybrids	Correlation coefficient
Kota bunt and Red Bobs bunt	Kota × Garnet	$+0.373 \pm 0.042$
Kota bunt and Red Bobs bunt	Kota × Red Bobs	$+0.082 \pm 0.059$
		Correlation ratios
Kota bunt and strength of straw	Kota × Red Bobs	-0.303 ± 0.036
Kota bunt and awning	Kota × Red Bobs	-0.262 ± 0.037
Red Bobs bunt and strength of straw	Kota × Red Bobs	$+0.204 \pm 0.051$
Red Bobs bunt and awning	Kota × Red Bobs	$+0.238 \pm 0.052$
Kota bunt and strength of straw	Kota × Garnet	-0.203 ± 0.041
Kota bunt and awning	Kota × Garnet	-0.235 ± 0.027
Red Bobs bunt and strength of straw	Kota × Garnet	$+0.191 \pm 0.043$
Red Bobs bunt and awning	Kota × Garnet	$+0.274 \pm 0.041$

Summary of correlation values. In Table XIV a summary is given of the correlation values derived in the course of the inheritance studies just reported. With the exception of one value, they may be considered significant. They

are, with the exception noted, of the same order and, while not large, may be considered highly significant for the purposes of the present study. Attention is drawn to the use by Harris (23), in his study of biological reactions in cotton, of similar small but statistically significant correlation coefficients.

Kota × Red Bobs hybrids. The correlation between the reactions of Kota × Red Bobs F_3 lines inoculated with bunt from Kota and from Red Bobs was $+0.082 \pm 0.059$. This non-significant value was expected in view of the susceptibility of both parents to both smuts. The wide scatter on this correlation surface suggested, however, that not all of the susceptibility found in the F_3 lines of these crosses was due to the same factors.

Kota × Garnet hybrids. In the Kota × Garnet F_3 lines the correlation between the reactions obtained by inoculation with the two original bunt collections is indicated by a coefficient of $+0.373 \pm 0.043$. Garnet is resistant and consequently there was a differential reaction among the various hybrid lines. The significant positive correlation indicates that the factor (or factors) for resistance in Garnet governs the reaction to both bunts.

As in the case of the Kota × Red Bobs lines further tests would have been necessary to prove the differential nature of certain F_3 lines that were very susceptible to Kota bunt and resistant to bunt from Red Bobs. It should be noted that no lines resistant to Kota bunt yet highly susceptible to Red Bobs bunt were found, although some lines were resistant to both kinds. The F_4 hybrid lines that were tested in 1931 came from a group comprised approximately of one-quarter of the total number of hybrid F_3 lines on which the double test had been made. They had shown in 1930 from 0 to 10% infection from Red Bobs bunt and 0 to 25% infection from Kota bunt and they continued to show some resistance in F_4 . A few lines showing considerable resistance will be tested further. As in the F_3 a significant positive correlation was found between the reactions of the two bunt collections used on the hybrids. In this case $r = +0.458 \pm 0.042$.

These findings bear out the earlier conclusions that Garnet resistance was operative against both bunts and would indicate that such resistance is conditioned by a single genetic factor.

Summary

The studies herein reported were made on the F_2 , F_3 and part of the F_4 population, in crosses between Kota and Red Bobs, and between Kota and Garnet. Duplicate populations in each cross proved to be similar in nature as shown by similarity in reaction to bunt and loose smut, by seed color segregation and by other characters studied.

Studies were made of inheritance of resistance to bunt from Kota and bunt from Red Bobs, of resistance to Kota loose smut, and of the characters, seed color, awns, and straw strength. Correlated inheritance studies were

also made of the reaction of the hybrids of the two crosses to the two bunt forms, and of the reaction of Kota bunt and Red Bobs bunt to awning and strength of straw.

Kota and Red Bobs were highly susceptible to both Kota bunt and Red Bobs bunt. Garnet was resistant to both bunts but less resistant to the Kota bunt.

The original bunt collections were considered to be essentially different in regard to physiologic reaction as well as in morphological character. The Kota bunt (*Tilletia levis*) was much more virulent than the Red Bobs bunt (mainly *T. tritici*).

The heritable nature of bunt reaction was clearly demonstrated. The percentage of infection found in the parent lines was recovered in the F_3 hybrids but no transgressive segregation was noted. An indication of the dominance of the bunt-resistance factors was obtained and the presence of several factors governing bunt reaction indicated.

Kota was relatively susceptible to loose smut, Garnet resistant, and Red Bobs immune under the conditions of test. The heritable nature of the reaction to loose smut apparently indicated by the close check in percentage of infection in the duplicate populations and by the relative percentages of infected lines from the F_3 of susceptible \times resistant and susceptible \times immune crosses, was not evident in the F_4 tests. Infection of the hybrid Kota \times Garnet population appeared to take place entirely in a random manner. It is suggested that such resistance as was apparent in the parental varieties might have other than a physiological basis.

Two factor pairs are believed to be concerned with segregation for color in the Kota \times Red Bobs hybrids. Since both parents are red it must be concluded that each carries a single factor pair for red color, unlike that carried by the other. Failure to obtain a good fit for the segregating classes in F_3 was not entirely explainable. Taken in conjunction with other findings in regard to seed coat color this study points to the need for a thorough investigation of this character with the practical end in view of obtaining homogeneity for color in our red wheat varieties.

The segregation observed for awn characters in the F_3 lines agreed with the results of Clark (6, 8, 9) from similar crosses. His method of awn classification (6) was used in preference to that of Stewart, but it was not found entirely satisfactory. It was concluded that more than one factor was probably involved in awn segregation in the Kota \times Garnet hybrids and at least two in the Kota \times Red Bobs hybrids.

Indications were obtained that a two-factor difference conditioned the segregation in strength of straw in the crosses of Kota \times Garnet. However, the complex segregation noted in this study of straw strength can best be explained on a multiple-factor basis. Transgressive segregation in the direction of greater weakness of straw was noted.

Correlation studies between the reactions of Kota \times Garnet hybrids to bunt from Red Bobs and Kota indicated that the same factor for resistance in Garnet governs the reaction to both bunts. Garnet resistance was found to be conditioned by a single partially dominant factor pair.

Considering the reaction of the two bunts used to inoculate the Kota \times Red Bobs hybrids it is noteworthy that, in both cases, susceptibility to bunt from a parent is associated with the strength of straw of that parent. Also in both cases the degree of resistance to the bunt from the parent is associated with the characteristic awn type of that parent. The conclusions to be drawn from the reactions of the two bunts with the Kota \times Garnet hybrids bear out the findings in regard to the Kota \times Red Bobs hybrids. Linkage between factors for strength of straw and susceptibility to bunt and a similar linkage between awn type factors and bunt resistance factors are evident.

Taken together the last-mentioned findings demonstrate the presence in wheat of two linkage groups concerned with the reaction to bunt forms. However, since the expression of both strength of straw and awn type appears to be conditioned by two or several factors it is believed that the linkage may involve only one pair concerned with each character. The correlation obtained, while significant, indicates that linkage is not complete in either case, nor can it be considered strong enough to interfere greatly with breeding for bunt resistance in wheat.

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PYOCYANINE AND GROWTH POTENTIAL CHANGES OF *PS. PYOCYANEUS*¹

By G. B. REED² AND E. M. BOYD³

Abstract

The pigment pyocyanine, produced by *Ps. pyocyaneus*, together with its leuco form has been shown by Friedheim and Michaelis to constitute a reversible oxidation-reduction system. Friedheim has shown that under certain circumstances the pigment may act as a respiratory enzyme. In this paper it is shown that two ordinary types of culture media, when sterile and freely exposed to the air, exhibit potentials more positive than that of pyocyanine. With the growth of *Ps. pyocyaneus* the potential, in the depths of the fluids, falls to a more negative value than that of the leuco form of this pigment. The surface layer exposed to air remains more positive than the potential of pyocyanine. The surface layer of the cultures therefore becomes colored; the deeper layers remain colorless.

These changes in potential in the culture fluid make ideal conditions for the functioning of pyocyanine as a respiratory catalyst—at the surface of the medium it is oxidized, in the depths reduced. The observation that a non pyocyanine-producing strain gives approximately the same growth-oxidation-reduction curves apparently indicates that the potential changes are not dependent on the pigment.

It was recently shown by Friedheim and Michaelis (6) and Michaelis (11) that the blue pigment, pyocyanine, produced by *Ps. pyocyaneus*, in mixture with its leuco compound constitutes a reversible oxidation-reduction system which at pH 6.0 and more alkaline reactions behaves like any of the known reversible dyestuffs of quinoid structure. Later Friedheim (5) demonstrated that the consumption of oxygen by washed cells of *Ps. pyocyaneus*, by other species of bacteria and by rabbit red cells, is increased in the presence of pyocyanine; the pyocyanine may act therefore as an accessory respiratory enzyme. A study of the oxidation-reduction potentials of growing cultures of *Ps. pyocyaneus*, which was in progress at the time the above papers were published, gives further evidence of the biological significance of this substance.

The Eh of completely oxidized pyocyanine at pH 7.25, according to Friedheim and Michaelis, is approximately +0.080 volts; that of the reduced form, the leucopyocyanine, -0.100 volts, and that of a mixture of equal parts of oxidized and reduced pyocyanine, -0.047 volts. Elema's (4) demonstration that from pH 6 to 9 the Eh of the pyocyanine system lies between that of methylene blue and indigo trisulphonate indicates in a less precise fashion the same reaction. Since pyocyanine can function as a respiratory catalyst only in a medium capable of potential change through this Eh range, the question arises as to whether, in ordinary cultures of *Ps. pyocyaneus*, such conditions exist. A series of growth-potential determinations were therefore undertaken.

The technique was similar to that described in a former paper, Boyd and Reed (3). More uniform results were obtained by using greater care in selecting

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and cleaning the platinum electrodes and in discarding those which deviated by more than 20 millivolts from the standard when tested in a solution of known potential. The culture-electrode vessels consisted of 250-cc. Erlenmeyer flasks stoppered with ordinary cotton plugs through which was passed the tube supporting the platinum electrode and one arm of a potassium chloride-agar bridge. The flasks were almost filled with fluid media, 200 cc., the platinum electrodes put in place near the bottom, and the cotton stoppers arranged with a removable glass rod in the opening for the potassium chloride-agar bridge. They were then autoclaved and, after cooling, the sterile agar bridges put in place. The necks of the flasks were large enough to allow free circulation of air through the cotton plugs. Cultures were incubated and tested at 37° C.

I

Flasks of beef extract broth containing 0.1 M phosphate as a buffer and adjusted to pH 7.2 were arranged as half-cells in the manner just described. After autoclaving they were allowed to stand for 48 hr. until the major positive

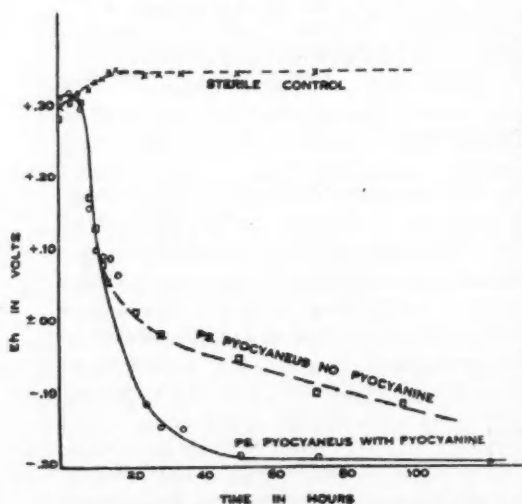


FIG. 1. Curves showing the oxidation-reduction potential of cultures of *Ps. pyocyaneus* growing in buffered broth: lower curve, a pyocyanine producing strain, data from Table I; middle curve, a non-pyocyanine producing strain, data from Table III; upper curve the sterile broth. Ordinates, Eh in volts; abscissas, time in hours.

drift in potential, consequent on the heating, had passed, in order that more uniform initial potentials in the different flasks might be ensured, as previously discussed by Boyd and Reed (2). The flasks were seeded with 0.1 cc. of a saline emulsion of organisms from a young agar culture. In each experiment three half-cells were inoculated and two similar cells without organisms were used as controls. The results of one such experiment are shown in Table I and the mean Eh values plotted in Fig. 1.

Growth was perceptible as a faint clouding of the medium in about eight hours. At this point, as indicated in Fig. 2, a precipitous drop in the potential began and continued for eight to ten hours to an Eh of approxi-

mately -0.10 volts. This was followed over a period of three weeks by a much more gradual decline to some -0.234 volts and by a slight positive drift during the fourth week. The culture fluid was sufficiently buffered that there was no change in pH. During this period the sterile broth controls remained practically constant at an Eh of +0.30 to +0.35 volts. Several series of cultures gave similar results.

The persistence of this low potential in the cultures for so long a period appears to be unique for cultures freely exposed to atmospheric air. Under similar circumstances cultures of streptococci (7), *B. diphtheriae* (8), staphylococci (9), and pneumococci (10) maintain low reducing potentials for only a few hours.

Pyocyanine first appeared in these cultures after 20-24 hr. as a faint blue-green coloration of the surface layer of the broth. The color gradually deepened to an intense blue-green but the colored material remained restricted

TABLE I
GROWTH POTENTIALS OF *Ps. pyocyaneus* IN BUFFERED BROTH AT pH 7.2

Time, hr.	Sterile controls			Cultures of <i>Ps. pyocyaneus</i>			
	1	2	Mean	1	2	3	Mean
	Eh, volts						
0	0.300	0.302	0.301	0.314	0.318	0.311	0.314
3	0.307	0.322	0.315	0.320	0.318	0.322	0.320
5	0.310	0.325	0.318	0.319	0.315	0.315	0.317
6	0.312	0.326	0.319	0.289	0.290	0.288	0.289
8	0.322	0.327	0.325	0.165	0.154	0.150	0.156
10	0.333	0.339	0.336	0.110	0.102	0.107	0.106
12	0.336	0.345	0.341	0.090	0.095	0.104	0.096
14	0.346	0.354	0.350	0.090	0.094	0.098	0.094
16	0.350	0.352	0.351	0.079	0.071	0.059	0.070
24	0.345	0.342	0.344	-0.100	-0.108	-0.123	-0.110
28	0.342	0.348	0.345	-0.132	-0.138	-0.152	-0.141
34	0.344	0.349	0.347	-0.140	-0.142	-0.150	-0.144
50	0.343	0.348	0.346	-0.178	-0.188	-0.188	-0.185
72	0.348	0.350	0.349	-0.187	-0.183	-0.188	-0.186
168	0.390	0.390	0.390	-0.192	-0.188	-0.208	-0.196
240	0.404	0.406	0.405	-0.204	-0.200	-0.225	-0.210
288	0.416	0.417	0.417	-0.221	-0.223	-0.234	-0.226
504	0.427	0.426	0.427	-0.223	-0.236	-0.245	-0.234
692	0.461	0.468	0.464	-0.188	-0.225	-0.166	-0.193

to the surface zone (1-2 cm. in thickness). This is in agreement with the results of the potential determinations. By the time enough pyocyanine had formed to perceptibly color the fluid, the potential in the depths, as determined by the electrodes placed near the bottom of the flasks, had fallen to an Eh of less than -0.10 which, according to Friedheim and Michaelis (6), is the potential of the leuco form of the pigment.

II

Ps. pyocyaneus grows luxuriantly on certain synthetic culture media, particularly the asparagin-glycerol-salt medium of Proskauer and Beck, commonly used for tubercle bacilli. To prevent changes in pH during growth, Proskauer and Beck's medium as modified by Kock (Baldwin, Petroff and Gardner (1)) was further modified by increasing the concentration of disodium hydrogen phosphate to ten grams per litre and adjusting the pH

with sodium hydroxide to 7.2.* Flasks containing this medium were arranged as half-cells, exactly as with broth, described in the previous section, inoculated from a young agar culture and the potential determined as in the previous instances. The results of one experiment are indicated in Table II and Fig. 2.

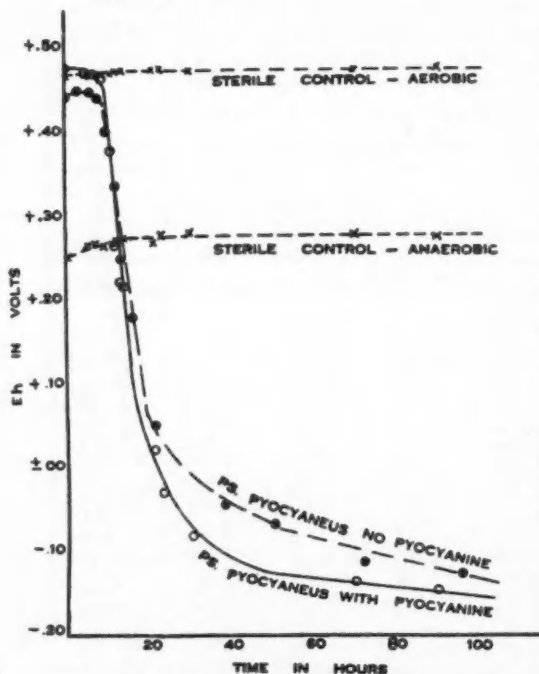


FIG. 2. Curves showing the oxidation-reduction potential of cultures of *Ps. pyocyaneus* growing in Proskauer and Beck's synthetic media: lower curve, a pyocyanine producing strain, data from Table II; second curve, a non-pyocyanine producing strain, data from Table III; two upper curves, the sterile medium under aerobic and anaerobic conditions. Ordinates, Eh in volts; abscissas, time in hours.

potential in the cultures, indicated in Table II and Fig. 2, must result directly from the metabolism of the bacteria.

Change in potential in the depths of cultures in this synthetic medium was similar to that in broth cultures. The precipitous fall began, as indicated in Fig. 2, nine or ten hours after the culture was inoculated and growth became perceptible as a faint clouding of the medium about two hours later. Both the conspicuous potential change and the gross indication of growth

*At this pH it is necessary to sterilise the medium in two parts in order to prevent the precipitation of magnesium phosphates. The magnesium sulphate and half the water is placed in one vessel, the other ingredients and the balance of the water in a second vessel. After autoclaving and cooling the two are combined.

With this medium two sets of sterile controls were used; one freely exposed to the air through cotton stoppers and one set with a vaseline seal over the surface of the fluid. It will be observed that in both instances the potential remained high and practically unchanged during the period of the experiment. This is in marked contrast with the behavior of sterile broth. Several observers have reported a fall in the potential of sterile broth under anaerobic conditions. In a former paper (2) evidence was presented which indicated that this results from the absorption of oxygen or other oxidizing bodies by the broth. It seemed possible that the fall in potential in the depths of growing cultures of *Ps. pyocyaneus*, and other bacteria, might result from similar changes. Since the aerobic and anaerobic sterile controls in this synthetic medium remain unchanged during the period of the experiment, it seems evident that the fall in poten-

TABLE II

GROWTH POTENTIALS OF *Ps. pyocyaneus* IN BUFFERED SYNTHETIC MEDIUM AT pH 7.0

Time, hr.	Sterile controls						Pyocyaneus, aerobic		
	Aerobic			Anaerobic					
	1	2	Mean	1	2	Mean	1	2	Mean
	Eh volts								
0	0.470	0.476	0.473	0.250	0.262	0.256	0.484	0.477	0.481
5	0.471	0.476	0.474	0.262	0.278	0.270	0.481	0.471	0.476
7	0.474	0.474	0.474	0.264	0.279	0.272	0.476	0.267	0.472
9	0.473	0.477	0.475	0.264	0.274	0.269	0.471	0.462	0.467
11	0.476	0.478	0.477	0.268	0.280	0.274	0.385	0.380	0.383
12	0.479	0.478	0.478	0.270	0.286	0.278	0.266	0.277	0.272
13	0.479	0.478	0.478	0.268	0.287	0.278	0.215	0.237	0.226
14	0.478	0.480	0.479	0.271	0.285	0.278	0.204	0.233	0.219
21	0.480	0.480	0.480	0.276	0.271	0.274	0.026	0.022	0.024
23	0.480	0.479	0.480	0.285	0.276	0.281	-0.036	-0.023	-0.030
30	0.478	0.478	0.478	0.292	0.281	0.287	-0.086	-0.075	-0.081
70	0.478	0.480	0.479	0.281	0.288	0.285	-0.133	-0.138	-0.136
90	0.477	0.483	0.480	0.272	0.291	0.282	-0.144	-0.151	-0.148
120	0.476	0.482	0.479	0.260	0.282	0.271	-0.137	-0.134	-0.136
168	0.475	0.482	0.479	0.264	0.276	0.270	-0.123	-0.134	-0.129
240	0.478	0.483	0.481	0.267	0.274	0.271	-0.141	-0.145	-0.143
288	0.478	0.484	0.481	0.270	0.280	0.275	-0.142	-0.147	-0.145
504	0.480	0.486	0.483	0.285	0.278	0.282	-0.143	-0.152	-0.148
692	0.484	0.488	0.486	0.260	0.284	0.272	-0.157	-0.155	-0.156

were therefore three to four hours later than in broth. The subsequent potential changes, it may be observed, were essentially the same as in the broth cultures although the maximum reducing potential in the synthetic medium was slightly less than in the broth. It is significant however from the point of view of the pyocyanine that an Eh of -0.10 volts was reached after 30-40 hr. growth and remained below this level for the period of the experiment, some four weeks, *i.e.*, below the level of oxidized or blue pyocyanine.

The blue pigment was apparent after 18-20 hr. growth, as in the case of the broth cultures, in a surface zone never more than a centimetre in thickness. The pigmentation gradually became more intense but remained restricted to the surface notwithstanding a heavy diffuse growth throughout the medium.

III

In the course of a study of the variation of *Ps. pyocyaneus* Reed (12) isolated a strain which produced no pyocyanine, though in other respects it was typical of the species. This strain was grown in broth and in the synthetic medium and the potential changes followed under the same conditions as described in two previous sections. The results of one such experiment are indicated in Table III and plotted in Figs. 1 and 2. It is evident that in both media the non pyocyanine-producing variant produced essentially the same

growth-potential curves as the typical pyocyanine-producing strains, although the final potential with the former was slightly less negative than in pigment-forming cultures. Cultures of both strains however assumed an Eh *more negative* than the Eh of leucopyocyanine.

It seems evident then that the oxidation-reducing potentials in cultures of this organism are not primarily due to pyocyanine.

IV

It is evident from these experiments that the two types of culture media, when sterile and freely exposed to air, give oxidation potentials which are more positive than the Eh of pyocyanine and, when under anaerobic conditions, the synthetic medium remains unchanged while broth gradually assumes a potential more negative than that of leucopyocyanine. Cultures of *Ps. pyocyaneus* in both media develop, in the depths, a potential more negative than that of leucopyocyanine, while the surface layer exposed to the air remains more positive than pyocyanine. As a result, in the case of

TABLE III
GROWTH POTENTIALS OF A NON-GREEN PYOCYANINE FORMING STRAIN OF *Ps. pyocyaneus* IN BUFFERED BROTH AND PROSKAUER AND BECK'S MEDIUM

Time, hr.	Growth potentials in buffered broth			Growth potentials in Proskauer and Beck's medium		
	1	2	Mean	1	2	Mean
	Eh volts					
0	0.276	0.295	0.286	0.422	0.474	0.448
3	0.309	0.311	0.310	0.430	0.489	0.460
5	0.316	0.319	0.318	0.430	0.488	0.459
6	0.311	0.307	0.309	0.431	0.482	0.457
8	0.177	0.177	0.177	0.430	0.459	0.445
10	0.143	0.121	0.132	0.406	0.401	0.404
12	0.072	0.084	0.078	0.380	0.301	0.340
13	0.049	0.070	0.060	0.257	0.245	0.251
16	0.083	0.087	0.085	0.184	0.182	0.183
21	0.017	0.016	0.017	0.024	0.082	0.053
28	-0.011	-0.016	-0.014	-0.033	-0.053	-0.043
50	-0.032	-0.066	-0.049	-0.055	-0.085	-0.065
72	-0.076	-0.092	-0.084	-0.096	-0.123	-0.110
96	-0.111	-0.113	-0.112	-0.112	-0.136	-0.124
168	-0.138	-0.154	-0.146	-0.104	-0.105	-0.105
336	-0.191	-0.193	-0.192	-0.121	-0.112	-0.117
504	-0.206	-0.204	-0.205	-0.092	-0.104	-0.098
672	-0.211	-0.212	-0.212	-0.073	-0.118	-0.096

the ordinary pyocyanine-producing strains, the surface layers become blue while the deeper portion of the fluid remains colorless. With diffusion therefore the pyocyanine will be alternately oxidized and reduced. This appears to constitute ideal conditions for the functioning of the pigment as a respiratory catalyst.

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THE CHEMICAL AND PHYSIOLOGICAL PROPERTIES OF CRYSTALLINE OESTROGENIC HORMONES¹

BY J. S. L. BROWNE²

Abstract

A crystalline oestrogenic hormone has been isolated from human placenta. This substance shows a melting point of 274° C. (uncorrected). The carbon-hydrogen analysis gives: carbon, 74.8%; hydrogen, 8.8%. The mixed melting point with theelol shows no lowering. The substance differs physiologically from previously isolated female sex hormones. It is relatively inactive on the adult ovariectomized albino rat. The dose required is 16 γ as compared with 1.5 γ for theelol. It is, however, effective on the immature intact rat, 21 days old, in a dose of 1.2 γ . This is the same as theelol. On the immature castrate, the dose of the placental crystals is 10 γ , that for theelol is 3 γ . The presence of the ovary is believed to be the factor in the dosage ratio between adult spayed and normal intact animals. Transplantation of immature ovaries into immature or adult castrates, even though it does not render them cyclic, causes the weight of the placental crystals needed for an effect to fall to the immature intact dose.

The isolation of a crystalline substance having oestrogenic properties was first reported by Doisy and coworkers in 1929 (22). Shortly after Butenandt also described the preparation of such a crystalline material (8). Marrian in 1930 reported the isolation of a crystalline substance having oestrogenic properties which he believed to differ from those of previously isolated crystals (24, 25). It has been shown by the work of Butenandt (9) and of Doisy and coworkers (34) that the first isolated substance called by Doisy "theelin" has an empirical formula of $C_{18}H_{22}O_2$ and contains one ketone and one hydroxyl group. The substance first isolated by Marrian (24, 25) and later by Butenandt (9, 10) and by Doisy and coworkers (20, 21, 33) has been shown to have an empirical formula of $C_{18}H_{24}O_3$, *i.e.*, to contain the elements of one more molecule of water than the keto-hydroxy form. It has three hydroxyl and no ketone groups. It has been called "theelol" by Doisy, and Marrian (26) uses the term "tri-hydroxy oestrin" to designate it. Butenandt (9, 10) has been able by heating the trihydroxy form in high vacuum with potassium acid sulphate to convert it to the keto-hydroxy form. In 1931 Marrian (26) on physiological grounds which will be discussed later suggested that two forms of keto-hydroxy oestrin existed. These he called "A" and "B" forms. He believed that the product obtained by Butenandt who used high vacuum distillation in his method was the "A" form and that Doisy's theelin was the "B" form. Butenandt and Störmer (11) in a paper just received have brought forward further evidence on both chemical and physiological grounds that two such isomeric forms exist. Their alpha form melts at 255° C. (uncorrected) and has an optical rotation in chloroform of $[\alpha]_D = 156-158^\circ$ and its benzoate shows a constant melting point at 217.5° C. (uncorrected). The β -hormone melts at 257° C. (uncorrected) has a rotation

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of $[\alpha]_D = 165-166^\circ$ and its benzoate shows two polymorphic forms, one melting at 220.5°C. and the other at 205°C. (both uncorrected). The greatest difference as discussed below lies in their physiological effect. Both forms are obtained on distilling the trihydroxy form in high vacuum, a slow distillation at $130-140^\circ \text{C.}$ and 0.02 mm Hg. tending to give the α -form and a rapid distillation at 200°C. tending to give the β -form. It is uncertain whether these isomers represent the "A" and "B" forms of Marrian (26) or not. Concerning the further structure of the oestrogenic substance Bernal (7) published evidence based on X-ray crystallography and Adam and coworkers (1) evidence based on surface films as an index of molecular dimensions tending to show that the crystalline oestrogenic substances possessed a structure like that of phenanthrene derivatives. Butenandt, Störmer and Westphal (12) in a very recent paper have reported extensive chemical investigations which lead to the conclusion that the phenanthrene derivative type of formula is the correct one. They offer tentative partial structural formulas and account for the presence of isomeric forms in the structure of the molecule. These formulas have been revised still more recently, and a four-ringed structure is now accepted.

Physiological Effect

The assay of these crystalline substances as to their oestrogenic effects has been performed almost wholly using the vaginal smear method on the ovariectomized adult white rat or mouse. There are many variable factors which affect the accuracy of this method of assay (2, 3, 6, 13, 23, 28, 29). The condition of the animal, as affected by age, nutrition, the presence of infection and the length of time since ovariectomy, are important in influencing the result obtained. Variation in individual susceptibility in animals has been emphasized by Coward and Burn (13) and others (2, 4, 5) and the necessity for use of large numbers of animals has been recognized. Variations in the method of administration, such as injecting in oil or water and subcutaneously or otherwise, play a role in governing the response. Butenandt (11) reports that when the dose is divided into four or six injections the injection medium has little effect on the result. Perhaps the most important assay factors are the degree of division of the total dose into separate fractions, the time interval between the injections and the length of time between assays. There is no general agreement as to the criterion of a positive response, some authors requiring a full squamous flush and some only a disappearance of leucocytes and the presence of large numbers of nucleated epithelial cells. The number of animals which must show such a positive response, before the amount injected is considered a unit, may be set at 50 or 75% or some other fraction of the total number in the test. The discovery of two forms of keto-hydroxy oestrin and Butenandt's finding that the trihydroxy form tends to form mixed crystals with the keto-form makes uncertain the variations in potency which may occur in different laboratories. As a result of differences in composition of the crystalline material all of these variations make it difficult to compare the values obtained for a unit in

different laboratories. Table I shows the weight of material in terms of γ (thousandths of a milligram) required for a unit by various authors for the different crystalline preparations.

The conditions of assay are given in summary. In this table the influence of division of the dose into several injections in Butenandt's assays on α follicular hormone is very obvious.

If the substance is given in four injections it requires only about one-fifth the dose for the same response as when given in a single injection. The difference in sensitivity between the rat and the mouse is difficult to deter-

TABLE I
ASSAY OF CRYSTALLINE OESTROGENIC SUBSTANCES

Form	Compound and author	Potency γ per unit	Animal	No. of injections	Time interval	No. of animals	Per- centage response per unit
Keto- hydroxy	Theelin (Doisy) (21)	0.02	A.M.	4 (W)	36	—	—
		0.38	A.R.	3 (W)	12	20	75
		1.08	I.R.	3 (W)	48	5	60
		1.08	I.R.	oral (W)	7	5	60
	Follicular hormone						
	Butenandt (9, 10, 11)	0.025	A.M.	6 (O)	48	10	80
	α -Form	0.03	A.M.	4 (O)	36	10	80
		0.07	A.M.	3 (O)	12	12	80
		0.125	A.M.	1 (O)	—	12	80
	α -Benzoate	0.100	A.M.	1 (O)	—	10	80
	β -Form	0.125	A.M.	6 (O)	48	10	80
		0.80	A.M.	1 (O)	—	10	80
	Oxime	2.00	A.M.	1 (O)	—	—	—
	Mono-acetate	0.167	A.M.	1 (O)	—	12	80
	Hydride	Inactive					
	Becker <i>et al.</i> (6)	0.38	A.M.	1 (O)	—	100	50
	Gustavson and d'Amour (4, 5)	0.38	A.R.	1 (O)	—	100	50
	Marrian (26)						
	Keto-hydroxy A	0.025	A.M.	4 (W)	36	20	50
	Keto-hydroxy B	0.060	A.M.	4 (W)	36	20	50
Tri- hydroxy	Marrian (25, 26)	0.13	A.M.	4 (W)	36	20	50
	Butenandt (10, 11)						
	Hydrate, m.p., 279° C.	13.3	A.M.	1 (O)	—	8	90
	272-273	10.0	A.M.	1 (O)	—	9	78
	266-268	4.0	A.M.	1 (O)	—	7	86
	263	2.5	A.M.	1 (O)	—	8	88
	Theiol (Doisy) (19, 22) (276° C.)	0.68	A.R.	3 (W)	12	20	75
		0.16	I.R.	3 (W)	48	5	60
		0.32	I.R.	oral (W)	—	5	—
		1.35-2.0	A.R.	oral (W)	—	7	—
	Emmenin crystals (274° C.)	16.0	A.R.	6 (W)	36	100	60
		1.55	I.R.	6 (W)	36	30	47

NOTE:—A = adult, ovariectomized; I = immature (normal); R = rat; M = mouse; W = water medium; O = oil medium.

mine. According to Becker *et al.* (6) the dose for the rat and mouse is the same. According to most other authors the mouse requires only about one-fourth the dose required by the rat. Recently Thayer and MacCorquodale (35) reported that the mouse was about 12 times as sensitive as the rat for theelin but had about the same sensitivity as the rat to theelol. In connection with Doisy's unit of 0.68 γ for theelol in the adult rat Thayer and MacCorquodale, working in Doisy's laboratory, found that the unit of theelol varied enormously according to whether the full squamous or the epithelial smear was required for a positive reaction. They found, on the other hand, that in the case of theelin there was little difference between the two criteria. In other words, all the rats which showed an epithelial response in the case of theelin later progressed to the squamous stage, but in the case of theelol assays, many rats reached an epithelial stage and then showed regression to a negative smear without ever passing through the squamous stage. Because of this finding the amount of theelol for a unit is considered by Doisy to be much greater than 0.68 γ (actually about 6.00 γ) when a full squamous flush is required before an animal is considered to show a positive reaction. The values of 1.08 γ for theelin and 0.16 γ for theelol, obtained for a unit by Doisy with the immature rat, are based on the opening of the vagina within 10 days of a first injection given at 19 days of age.

As seen in the table and as mentioned above, Marrian reported in 1931 (26) that there are two forms of keto-hydroxy oestrin. He also believes that the first and more active form A is obtained when the substance is purified by high vacuum distillation according to Butenandt's method. He believes that the A form changes into the B or less active form on standing even in the dry state. The two forms are also distinguished by differences in the time after injection at which the greatest number of positive responses appear. The A form is slower and more prolonged in its action. The results in Butenandt and Störmer's recent paper make the occurrence of two forms of keto-hydroxy oestrin more certain. The difference of physiological activity between the two forms is seen in the table. The α -form is about six or seven times more active than the β -form. Butenandt is not certain of the possibility of interconversion of the α - and β -forms. He reports that the α -form occurs only in human pregnancy urine and that most of the oestrogenic activity in the urine of pregnant mares is of the β -form. Certain difficulties arise when one compares the results from different laboratories. Thayer and MacCorquodale (35), working in Doisy's laboratory, have recently shown that by Marrian's technique of assay on the mouse, theelin after keeping for from one week to eight months assays at less than 0.02 γ per unit. This is as potent as Butenandt's α -form. D'Amour and Gustavson (4, 5) who prepare their product by distillation at 0.02 mm. and 130-140° C. should, under these conditions, obtain the α -form, obtain a product which shows the same unit on the mouse and rat and gives for both a unit of 0.38 γ . Butenandt (11) finds that for a single injection in oil the α -form of keto-hydroxy oestrin causes a much less prolonged response in the animals than does the tri-

hydroxy form. Marrian (26), on the other hand, finds that the α -form causes a very prolonged response which occurs after a long latent period when he injects an aqueous solution in four doses over 36 hr. and that the trihydroxy form gives an earlier and shorter response. In this laboratory, on injecting an aqueous solution in six doses in 36 hr., it has been found that theelol and emmenin give a reaction which in its time relations resembles the results of Marrian on trihydroxy oestrin. The reaction with theelin begins slightly later and lasts slightly longer. The latent period and duration in the case of theelin however are not comparable to those obtained by Marrian on a keto-hydroxy oestrin. In 1931 Collip (17) reported the isolation by the present author of a crystalline substance from human placenta using Marrian's method described for pregnancy urine. These crystals showed a similar melting point to Marrian's preparation but were found to have a very low potency when assayed on the adult castrate rat by injection and to be active in doses of 1 γ in immature rats by oral administration. For the trihydroxy form Butenandt and Hildebrandt (10) found that crystals prepared according to Marrian's method (24, 25), melting at 269° C., showed a weight for a unit of 0.68 γ when given in a single injection and 0.12 γ when given in four injections. This last value is in good agreement with Marrian's own value of 0.13 γ shown in the table. However by another method Butenandt was able to prepare a substance having the same elementary analysis and group analysis as the first preparation. This form of trihydroxy oestrin melted at 276° C. or slightly higher than Marrian's preparation. However no lowering of melting point was obtained when the two preparations were mixed and they showed the same specific rotation. The preparation melting at 276° C. showed a weight of 5-10 γ for a unit, or was only one-seventh to one-twelfth as active as the first preparation. Recently Butenandt and Störmer (11) have obtained further results on trihydroxy oestrin. The lower the melting point of the preparation the higher its potency. This they have shown to be due to the contamination of the trihydroxy form with the keto-hydroxy form. Removing the keto-hydroxy form by formation of the semi-carbazone, which is insoluble under the conditions of their experiments, they have isolated from these various preparations a substance which has a potency of 75,000 mouse units per gram (13.3 γ for a unit). The melting point of this substance has been raised to 279° C. (uncorrected). This substance they regard as the pure hydrate uncontaminated with the keto-hydroxy form. In a preliminary report by Collip, Browne and Thomson (18) the possibility of a conversion of the emmenin and theelol to some more active form of oestrogenic substance within the organism and the influence of the ovary in facilitating this conversion was discussed. From the time relations of the animal responses to the pure hydrate Butenandt and Störmer also suggest that even the low potency remaining is due to its partial conversion in the organism to the keto-hydroxy form. Butenandt believes that most of the preparations of trihydroxy oestrin have been mixed with the keto-hydroxy form. As far as the melting point goes there is little difference in potency between that melting at 279° C. and that at 273° C. especially

when the small number of animals used and the percentage response is considered. Moreover Marrian (26, 27) has raised the melting point of his preparation, using an Anschütz thermometer, to 280° C. without reducing its potency. Thayer and MacCorquodale (35) were unable to change the potency of theelol by treatment with hydroxylamine to form the oxime of any theelin present (the oxime as shown by Butenandt has a low potency). They were unable to detect the presence of any theelin in theelol when they attempted to form the semicarbazone of the former. This contrasts with the impure forms of trihydroxy oestrin from which Butenandt readily isolated the semicarbazone of any keto-hydroxy form present. It is difficult to compare Doisy's assays on theelol with those of Butenandt on the hormone hydrate. Doisy's latest figure for theelol, reported at the meeting of the American Society of Biological Chemists in April, 1932, is a unit of about 6.00 γ when injected three times in 12 hr. in the adult ovariectomized rat requiring a squamous response in 75% of the animals. The possibility of the existence of trihydroxy oestrin in more than one form is admitted by Butenandt.

Experimental

In 1930 Collip (14, 15, 16) reported the presence in a crude extract of human placenta of a substance having chemical and physiological properties differing from those of oestrin as then known. This oestrogenic principle was characterized by its non-extractability by ether from acid aqueous solution and by its inactivity in producing oestrus in the adult ovariectomized animal when given by injection. It was, however, very effective when given by mouth to immature intact rats. Later, by using a larger number of animals and dividing the dose into several injections over a period of 36 hr. it was found that the substance was active in producing oestrus when given in large amounts to adult ovariectomized animals but was effective in a fraction of this dose when given to immature intact animals either by feeding or by injection. This oestrogenic substance was given the name emmenin. In the course of its preparation thorough extraction of the solution was performed in order to remove all the ether-soluble oestrogenic substances from the ether-insoluble emmenin. The present author, using the ether extract obtained in this manner, was able to isolate a crystalline substance by a procedure which followed that of Marrian (24, 25) for the preparation of trihydroxy oestrin from pregnancy urine (17, 18). The properties of this crystalline substance obtained from human placenta, the evidence for its identity with the active principle responsible for the emmenin type of oestrogenic activity and a comparison of its physiological properties with those of other crystalline substances, theelol and theelin, will be discussed in this paper.

Throughout this article the placental crystalline substance will be referred to, for convenience, as "emmenin". But if our suggestion that these crystals are a pure trihydroxy oestrin is acceptable, the name emmenin becomes redundant in this connection, though it may still fittingly be employed to

refer to the original crude ether-insoluble fraction, in which, it is believed, the trihydroxy oestrin occurs in combination with some substance unknown which affects its solubility.

Chemical and Physical Properties

Through the kindness of Drs. Graham and Osborne of the Department of Mineralogy a partial crystallographic examination of this preparation was carried out. The system of the crystals was monoclinic, the habit bladed, and they were optically positive. The crystals show an inclined extinction of 7° . Approximate refractive indices were α 1.54, γ 1.68. The crystallographic properties of Doisy's theelol as determined by Slawson (32) are quite comparable with these except that the crystals seem to be optically negative.

The best preparations of the crystalline emmenin melt fairly sharply at 274° C. (uncorrected) with preliminary darkening at 270° C. Through the kindness of Dr. Doisy in supplying a sample of his theelol it has been possible to compare it with emmenin. The sample of theelol melted at 276° C. with slight preliminary darkening. A mixture of equal parts of the two preparations melted at 275° C. (uncorrected). The carbon-hydrogen analysis of two samples of emmenin was: C, 74.8; H, 8.8 and C, 74.8; H, 8.9% respectively.* The crystals are soluble to about 0.05% in boiling water. As far as this chemical evidence goes these crystals appear to be very closely related to theelol. Theelol and emmenin however differ widely in their physiological properties.

We are deeply indebted to Dr. Doisy for supplying us with samples of theelol and theelin, the physiological properties of which we have thus been enabled to compare with those of emmenin.

The probable identity of the crystalline substance with the active principle present in the crude ether-insoluble emmenin fraction is indicated by the following considerations.

Dr. Collip has been able by autoclaving the crude emmenin fraction, obtained by precipitation with ammonium sulphate, at 150 lb. pressure for several hours, to render the active material entirely ether-soluble and to isolate crystals from the emmenin fraction of pregnancy urine by treatment of the ether extract so obtained. These crystals are indistinguishable physiologically from those obtained from placenta by the author. The crystals from pregnancy urine melted at 275° C. (uncorrected) and gave the same carbon-hydrogen analysis. Dr. Collip has also been able to prepare theelol from pregnancy urine. These crystals showed the same physiological properties as those of the samples supplied to us by Dr. Doisy.

The theelol, theelin, and crystalline emmenin from human placenta were compared as to their effectiveness in producing vaginal oestrus in the white rat. Various classes of animals were used.

1. The adult ovariectomized rat.
2. The immature intact rat.

*Research Service Laboratories, New York.

3. The immature intact rat (using oral administration).
4. The immature ovariectomized rat.
5. The immature ovariectomized rat with two immature ovaries transplanted into the spleen.

In assaying emmenin the total amount was injected subcutaneously in aqueous solution in six doses over 36 hr. A full squamous smear or one in which the number of squamous cells greatly exceeded the number of nucleated epithelial cells was called a squamous response. The number of animals showing an absence of leucocytes and the presence of large numbers of nucleated epithelial cells was noted and this number added to those showing a squamous response. The percentage of rats showing either a squamous or an epithelial response is given in the last column of the tables. The definition of these conditions of assay is important since variations in the number of injections and in the criterion of a positive response make great differences in the results obtained.

The Adult Ovariectomized Rat

Table II shows the comparison of the action of emmenin, theelol and theelin using the adult ovariectomized animal as the test object. The dose of

TABLE II
COMPARISON OF EFFECTS OF CRYSTALS OF EMMENIN AND CRYSTALLINE THEELOL AND THEELIN ON ADULT OVARECTOMIZED RATS BY INJECTION SIX TIMES IN 36 HR.

Group	Treatment	Dose γ	Total number of animals	Number of animals giving response indicated			Per cent response	
				Full sq.	Epith.	Neg.	Sq.	Sq. and epith.
1. Old castrates	Emmenin 15	11.0	28	6	1	21	21	25
	Emmenin 10	13.5	21	7	6	8	33	62
	Emmenin 15	20.5	28	15	6	7	54	75
	Emmenin 15	20.5	12	9	1	2	75	83
	Emmenin 15	14.6*	13	0	4	9	0	31
	Theelol	2.5	15	8	2	5	53	67
		3.0	14	6	1	7	30	50
		5.0	21	17	3	1	81	95
2. Recent castrates	Emmenin 15	15.7	100	62	20	18	62	82
	Theelol	1.57	100	80	8	12	80	88
3. Sensitized castrates	June 13 Theelol	1.44*	47	6	3	38	12	19
	June 20 Theelol	1.72	46	31	3	12	70	75
	June 27 Emmenin 15	7.20	40	8	12	20	20	50
	July 4 Emmenin 20	7.50	46	17	9	20	37	56
	July 12 Theelol 2	2.68	44	25	2	14	57	61
4. Recent castrates (rats not used previously)	May 30 Theelin	0.15	22	0	0	22	0	0
	June 6 Theelin	0.36	17	1	0	16	6	6
	June 13 Theelin	0.90	25	22	0	3	88	88
	June 20 Theelin	0.40	29	27	1	1	93	96
	June 27 Theelin	0.15	29	0	0	29	0	0
	July 4 Theelin	0.30	29	5	1	23	18	21

*These rats had not been used for a month before this experiment.

emmenin required for a given response varies according to the condition of the animal. The first group of rats had been ovariectomized some months before these assays and had been used from time to time. All the groups except that on the 14.6 γ dose had been primed with theelol before use. The lower response of the unprimed rat is evident. From these assays emmenin seems to be about one-fifth as active as theelol. In Group 2, a large group, the animals had been ovariectomized two weeks before the first emmenin assay and no previous priming dose was given. One week later the dose of 1.57 γ of theelol produced an 80% squamous response. In this assay emmenin is less than one-tenth as active as theelol though the unit for both is less than in the case of the old castrates. The objection might be raised that the first dose of emmenin sensitized the rats to the theelol and that if the theelol had been given first the dose for both would have been the same for both substances. In Group 3 the influence of six weeks rest on the response of the animals is seen. These are part of the group of 100 mentioned above. One week later a 70% squamous response was obtained with 1.72 γ of theelol. The next week 7.20 γ of emmenin gave a 20% response and another preparation of emmenin a 37% response. Finally a second preparation of theelol, also kindly supplied by Dr. Doisy, gave a 57% response with 1.68 γ on the same group of animals.

The above experiments show that emmenin is approximately one-fifth as active as theelol when tested on the adult ovariectomized animals under these conditions of assay. This physiological difference contrasts with the close chemical relation. The results with theelin are incomplete but indicate the effect of a previous larger dose on sensitizing the animals to a subsequent much smaller dose. These rats were recently castrated and were used only for theelin assay.

The Immature Intact Rat

The immature intact rat of 21 days of age responds in quite a different manner. The results as shown in Table III demonstrate that emmenin acts in a much lower dose in the immature intact animal than in the adult ovariectomized animal, that theelol acts in the same dose in both types of animal, and that emmenin and theelol are equally effective on the immature rat. There is considerable individual variation in the response of different groups of animals. Emmenin preparation No. 20 (m.p., 270° C.) seems slightly more active than preparation No. 15 (m.p., 274° C.). It was also slightly more active on the adult ovariectomized animal.

The effect of theelin on the immature rat is seen in the third part of Table III. It seems to be slightly more effective than emmenin or theelol but if one compares emmenin No. 20 and theelin, 0.92 γ of the emmenin gives a 33% response, 0.90 γ of theelin gives a 50% squamous response and 1.40 γ of emmenin gives a 100% response as does 1.20 γ of theelin. Compare this with the action on the adult where after a previous sensitizing dose in each case emmenin No. 20 gave a 37% response on 7.5 γ whereas theelin gave a 93% response on 0.40 γ . In other words emmenin was about one twenty-fifth as active as theelin in the adult ovariectomized rat.

TABLE III

COMPARISON OF EFFECTS OF CRYSTALS OF EMMENIN AND CRYSTALLINE THEELOL AND THEELIN ON IMMATURE INTACT RATS BY INJECTION SIX TIMES IN 36 HR.

Treatment	Age	Dose γ	Total number of animals	Number of animals giving response indicated			Per cent response	
				Full sq.	Epith.	Neg.	Sq.	Sq. and epith.
Emmenin	22	0.92	6	2	1	3	33	50
Ext. 20	22	1.13	10	0	0	10	0	0
15	22	1.21	13	7	3	4	54	70
15	22	1.32	12	3	1	8	25	33
20	22	1.40	13	12	1	0	93	100
15	22	1.55	30	14	4	12	47	60
15	22	1.65	10	0	1	9	0	10
10	22	1.80	6	3	1	2	50	67
Theelol	22	1.25	6	3	0	3	50	50
	22	1.62	9	4	5	0	45	100
	22	2.50	6	4	0	2	68	68
Theelin	22	0.45	10	0	0	10	0	0
	22	0.60	16	1	3	12	6	25
	22	0.60	15	4	2	9	26	40
	22	0.90	6	3	0	3	50	50
	22	1.20	9	9	0	0	100	100
Effect of repetition of the dose	24	0.60	16	1	3	12	6	25
	31	0.60	16	10	1	5	62	69

The Immature Intact Rat (Oral Administration)

The activity of emmenin and theelol is also the same when they are given orally to the immature intact rat as shown in Table IV. The total dose was given over three days and was placed in the drinking cup so that a slow, almost continuous absorption took place. In agreement with the finding

TABLE IV

COMPARISON OF EFFECTS OF CRYSTALS OF EMMENIN AND CRYSTALLINE THEELOL AND THEELIN ON IMMATURE INTACT RATS. EFFECT OF ORAL ADMINISTRATION

Treatment	Dose γ	Total number of animals	Number of animals giving response indicated			Per cent response	
			Full sq.	Epith.	Neg.	Sq.	Sq. and epith.
Emmenin 15	0.92	10	3	3	4	30	60
15	1.00*	20	2	3	13	10	35
10	1.26	9	5	2	2	55	75
10	1.40	6	3	1	2	50	67
10	1.80	5	3	0	2	60	60
15	2.00*	20	3	2	13	15	35
Theelol	1.32	10	3	0	7	33	33
	1.50	5	5	0	0	100	100

*These assays were on the same group of animals.

that division of the dose into parts increases the effectiveness of oestrogenic substances both emmenin and theelol are more effective when administered by mouth in this way than by injection when the dose is given in six parts in 36 hr. Variation in groups of rats is again seen in this table.

The Immature Ovariectomized Rat

Because of weight differences and possible differences in absorption from the subcutaneous tissue the comparison between adult ovariectomized animals and immature intact animals is perhaps not quite valid. The use of the immature ovariectomized animal of about the same age as the intact normal used above overcomes this difficulty. The results are strikingly different from those with the immature intact animal, and are shown in Table V. The dose of emmenin required is about the same or even slightly greater than that for a corresponding response in the adult ovariectomized animal. The dose of theelol required for the immature ovariectomized rat is about one and a half times that for the immature intact rat.

TABLE V

COMPARISON OF EFFECTS OF CRYSTALS OF EMMENIN AND CRYSTALLINE THEELOL AND THEELIN ON IMMATURE OVARIECTOMIZED RATS BY INJECTION SIX TIMES IN 36 HR.

Treatment	Age at castration	Age at injection	Dose γ	Total number of animals	Number of animals giving response indicated			Per cent response	
					Full sq.	Epith.	Neg.	Sq.	Sq. and epith.
Emmenin 15	21	23	4.4	11	1	0	10	9	9
	21	23*	6.2	11	2	2	7	18	36
	21	23	9.5	10	2	3	5	20	50
	21	29*	10.0	11	4	1	6	36	45
	21	50	10.2	15	11	2	2	73	87
	21	23	12.0	10	7	1	2	70	80
Emmenin 10	28	31	6.0	8	3	1	4	37	50
Theelol (repeated doses)	21	$\begin{cases} 26 \\ 32 \end{cases}$	$\begin{cases} 2.43 \\ 3.24 \end{cases}$	$\begin{cases} 20 \\ 20 \end{cases}$	$\begin{cases} 0 \\ 11 \end{cases}$	$\begin{cases} 1 \\ 2 \end{cases}$	$\begin{cases} 19 \\ 7 \end{cases}$	$\begin{cases} 0 \\ 55 \end{cases}$	$\begin{cases} 5 \\ 65 \end{cases}$
	19	$\begin{cases} 21 \\ 32 \end{cases}$	$\begin{cases} 1.62 \\ 2.43 \end{cases}$	$\begin{cases} 6 \\ 12 \end{cases}$	$\begin{cases} 1 \\ 6 \end{cases}$	$\begin{cases} 0 \\ 2 \end{cases}$	$\begin{cases} 5 \\ 4 \end{cases}$	$\begin{cases} 16 \\ 50 \end{cases}$	$\begin{cases} 16 \\ 66 \end{cases}$
Theelin (repeated doses)	22	26	0.30	7	0	0	7	0	0
	22	24	0.60	13	2	3	8	15	38
	22	26	0.60	8	2	0	6	25	25
	22	$\begin{cases} 26 \\ 30 \end{cases}$	$\begin{cases} 0.60 \\ 0.75 \end{cases}$	$\begin{cases} 8 \\ 8 \end{cases}$	$\begin{cases} 2 \\ 7 \end{cases}$	$\begin{cases} 0 \\ 0 \end{cases}$	$\begin{cases} 6 \\ 1 \end{cases}$	$\begin{cases} 25 \\ 89 \end{cases}$	$\begin{cases} 25 \\ 89 \end{cases}$
	22	$\begin{cases} 26 \\ 30 \end{cases}$	$\begin{cases} 0.30 \\ 0.75 \end{cases}$	$\begin{cases} 7 \\ 7 \end{cases}$	$\begin{cases} 0 \\ 7 \end{cases}$	$\begin{cases} 0 \\ 0 \end{cases}$	$\begin{cases} 7 \\ 0 \end{cases}$	$\begin{cases} 0 \\ 100 \end{cases}$	$\begin{cases} 0 \\ 100 \end{cases}$
	21	$\begin{cases} 23 \\ 35 \\ 42 \end{cases}$	$\begin{cases} 0.60 \\ 0.60 \\ 0.40 \end{cases}$	$\begin{cases} 13 \\ 13 \\ 13 \end{cases}$	$\begin{cases} 2 \\ 11 \\ 4 \end{cases}$	$\begin{cases} 1 \\ 0 \\ 2 \end{cases}$	$\begin{cases} 10 \\ 2 \\ 7 \end{cases}$	$\begin{cases} 15 \\ 84 \\ 31 \end{cases}$	$\begin{cases} 23 \\ 84 \\ 46 \end{cases}$

*This is the same group of rats.

Thus the difference between the activity of emmenin on the adult ovariectomized and immature intact rat is not related to weight differences or differences in absorption rate in the two types of test object. The difference in response in the immature intact and immature normal animal suggests that the presence of the immature ovary plays some role in the greater sensitivity of the normal animal. That the operation itself has no effect on the response of an animal to emmenin is shown by a control experiment where six animals were operated upon at the age of 21 days. The uteri were pulled on and cut but the ovaries were not removed. These animals showed a 100% squamous response on 1.5 γ of emmenin No. 15.

Theelol appears to be slightly more effective in the presence of the ovary but the difference is not nearly as great as in the case of emmenin.

The difference of effect between the normal and ovariectomized immature rats tends to decrease as the number of injections into which the total dose is divided is increased. Thus when nine injections in 48 hr. are given, a dose of 6.2 γ of emmenin is sufficient to give an 88% squamous response. And when the extract is fed over three days by the method mentioned above there is no difference between the response of the ovariectomized and of the intact immature animal.

The effect of theelin on the immature ovariectomized animal is the same or greater than its effect on the intact rat of the same age when it is injected in six doses over 36 hr. The sensitizing action of one dose of theelin on the next is very marked. Thus one group of 13 animals gave a 15% squamous response to 0.60 γ . Twelve days later they showed an 84% response to the same dose, and one week later a 31% response to 0.40 γ . In the case of emmenin this sensitization is not seen, a group of 11 immature ovariectomized rats showing an 18% response to 6.2 γ and six days later a 36% response to 10.0 γ . This is only the increase to be expected with the increase in dosage.

The Immature Ovariectomized Rat (With Two Immature Ovaries Transplanted)

In an endeavor to elucidate further the possible role of the ovary in the change of sensitivity to emmenin observed when the immature rat was ovariectomized, transplantation experiments were undertaken. The rats were castrated, usually at 21 days of age, tested for their response to one of the oestrogenic substances, and six days later received two ovaries from 21-day-old rats. These were transplanted into the spleen of the recipient. Two days later the rats were again tested with the same oestrogenic substance as before. The results are shown in Table VI.

The transplantation of the ovaries into immature ovariectomized rats lowered the dose of emmenin and theelol required for a given response to the level for the immature intact animal. This is further evidence for the theory that the immature ovary plays a specific role in the difference in response to emmenin observed in the ovariectomized and intact animal. That implantation of the ovaries alone does not produce a positive reaction is shown by the results on a group of nine 23-day normal rats which received two adult ovaries in the spleen. The smears were followed daily, one showed a positive

TABLE VI

COMPARISON OF EFFECTS OF CRYSTALS OF EMMENIN WITH CRYSTALLINE THEELOL AND THEELIN ON IMMATURE RATS. TRANSPLANTATION EXPERIMENTS

Treatment	Test animal	Age	Dose γ	Total number of animals	Number of animals giving response indicated			Per cent response	
					Full sq.	Epith.	Neg.	Sq.	Sq. and epith.
Emmenin	Group 1 Castrated	19							
		23	6.2	11	2	2	7	18	36
		28	10.0	11	4	1	6	36	45
	Transplanted	30							
		34	2.7	11	10	1	0	91	100
		41	1.8	11	7	3	1	63	91
Emmenin	Group 2 Castrated	21							
		24	9.5	10	2	3	5	20	50
	Transplanted	27							
		30	2.3	10	7	2	1	70	90
		36	1.56	10	6	1	3	60	70
Emmenin	Group 3 Castrated	21							
		23	12.0	10	7	1	2	70	80
	Transplanted	26							
		29	1.35	10	8	0	2	80	80
Theelol	Group 1 Castrated	23							
		26	2.43	20	0	1	19	0	5
		32	3.24	20	11	2	7	55	65
	Transplanted	35							
		37	1.72	20	15	1	4	75	80
Theelin	Group 1 Castrated	21							
		26	0.60	15	4	2	9	26	40
		28	0.60	14	12	1	1	86	93
	Transplanted	34							
		36	0.40	13	10	2	1	77	94

smear at the age of 51 days, five were cyclic at 60 days of age. Ten immature rats were ovariectomized at the age of 19 days and received two ovaries from 19-day-old rats when the recipients were 28 days old. The smears were read daily. No positive smear was noted up to three weeks after the implantation. The rats were 85 days old when the readings were stopped and only one was cyclic. This may be due to degeneration of the grafts or to the rats being still immature. In this connection one of a group of rats which had been implanted was killed a month after implantation. The rat was acyclic and the implanted ovaries were visible on the spleen. The graft weighed 14 mg.,

the original implant 7 mg. The graft consisted apparently of immature follicles. Another group of ten rats was ovariectomized at 20 days, transplanted with 21 day ovaries at 27 days and the smears followed daily. One showed a positive smear at the age of 40 days and remained cyclic, and one became cyclic at the age of 84 days. The results indicate that the transplantation of immature ovaries into immature ovariectomized rats will not of itself cause a positive smear within the term of the transplantation experiments described above. The results of Martins and Rocha (30) on parabiosis of castrate immature males with immature normal females, where the immature female became cyclic, indicate that the immature castrate male shows certain changes (presumably in the anterior pituitary) which stimulate an immature ovary brought under the influence of substances in the circulation of such an animal by parabiosis. It was thought that the transplantation results might be accounted for on the basis of changes occurring in the pituitary of the immature female after castration—such as the occurrence, when the ovaries were later implanted into the animal, of a premature stimulation of these ovaries with the production of an oestrus cycle.

The mechanism of action of the immature ovary in producing this increase of sensitivity to emmenin is not clear. The immature ovary might contribute part of the amount of oestrogenic substance required from its own store of this type of substance either because at this period after implantation the graft has not fully taken and part of the implant is being resorbed or because, the graft having taken, the immature ovary is being stimulated to secrete a certain amount of oestrin into the circulation. It has been shown in this laboratory that macerated immature ovaries implanted into the adult ovariectomized animals produced a positive response in a dose of 40 mg. The usual weight of two immature ovaries is 7-10 mg. The possibility of a pre-pubertal secretion of oestrin even in the intact rat has been noted by Mirskaia and Wiesner (31). According to this point of view the dose of emmenin is less for both the immature intact and the immature implanted animal because the ovary is in each case contributing part of the required amount of oestrogenic substance and hence the amount which must be injected is less. A control experiment was performed in this connection. Ten immature rats 21 days old were implanted with two extra ovaries from 21-day-old rats. They were injected two days later with 0.83 γ of emmenin 15, which on ordinary immature rats showed a 54% positive response on 1.21 γ . One of the rats with the extra ovaries showed an epithelial response and the rest were negative. The presence of two extra ovaries does not therefore make the rats more sensitive to emmenin, as would be expected if the implanted ovaries were contributing some of the oestrogenic substance required. The fact that the dose of theelin required for the immature normal rat is the same as for the immature ovariectomized rat is also against the view that the effect of transplantation on the sensitivity to emmenin is due to the immature ovary contributing a part of the dose of oestrogenic substance required. The effect of transplantation on the dose of theelin required is not so easy to determine on account of the marked sensitizing effect of one dose of theelin

on the next. One cannot tell whether any increase in sensitivity of the rats after transplantation is due to the effect of the transplantation or to that of the dose of theelin given before transplantation. Further experiments are needed in this connection. A group of 15 animals was given 0.06 γ of theelin at the age of 21 days, and showed a 26% response. They were ovariectomized at the age of 26 days and two days later were tested again with 0.60 γ . They showed an 86% positive response. This may be compared with the increase in sensitivity on repeating the dose of 0.60 γ of theelin on another group of 16 rats which were not ovariectomized between the tests but where the interval between the assays was the same. In the latter group the first test gave a 6% and the second a 62% positive response. There is no significant difference between the two groups so that ovariectomy seems to make little difference to the increase in sensitivity due to a previous dose of theelin. The absence of effect of ovariectomy is also seen in the last group in Table VI. The rats in this group were ovariectomized before the first dose of theelin. The first group of animals received two 21-day-ovaries at the age of 34 days. Two days later they showed a 77% positive response with 0.4 γ of theelin. This result may be compared with the last group in Table VI. Here the response of animals ovariectomized at 21 days was 15 to 0.60 γ at 23 days of age; at 35 days of age it was 84% to the same dose, and at 42 days of age 31% to 0.40 γ . In this case the transplantation of two ovaries seems to have increased the sensitivity of the animals in the first group as compared to that of the second group which received the same number of doses of theelin at the same intervals previously, but in which no transplantation was performed. The increase in sensitivity is slight, however, when compared with that occurring when emmenin is used on animals before and after transplantation and the number of animals is too small for any conclusion to be drawn.

Adult ovariectomized animals have also been used for transplantation experiments. Immature ovaries were used. The smears were followed daily. The danger of the occurrence of spontaneous oestrus makes these results less reliable than those on the immature animal. A large number of the old adult animals thus implanted remain acyclic. However most of them now gave a positive response to the immature normal dose of emmenin (1.5 γ) where before they required 7-16 γ for a similar response. This is taken to indicate that the presence of the implanted ovary, even though the graft may from outward appearances have degenerated (since no cyclic phenomena are produced), is still capable of making the animals respond to the immature normal dose of emmenin.

While there are still many obscure features it is felt that the theory which at present is most tenable, in view of the above considerations, is that the immature ovary may convert emmenin and probably theelol to some more active form of oestrogenic substance (probably some form of keto-hydroxy oestrin). This conversion is not impossible in the absence of the ovary but the rate of conversion and of utilization of emmenin is greatly increased in

its presence. The adult ovary also may presumably perform this conversion but it is not necessary that the ovary be producing cyclic changes for this conversion to occur.

The length of time since transplantation in the above experiments, necessarily limited because of the danger of spontaneous puberty later, makes it uncertain whether any effect is due to a secretion by the implanted ovary in the true sense after the graft has taken, or whether it is due to the substances which effect the conversion of emmenin being liberated from the graft before it has actually taken. The experiments with the adult implanted animals indicate that even after some weeks, when presumably a blood supply has been established, the conversion effect takes place.

The latest work of Butenandt (11) shows that trihydroxy oestrin uncontaminated with the keto-hydroxy form has a potency, when assayed on the adult castrate mouse by a single injection in oil, of 75,000 units per gram, requiring 80% approximately of the test animals to show a positive response. It is very difficult to compare this with the values with emmenin because of assay differences.* Emmenin has a potency of 50,000-70,000 units per gram when an 80% positive response is required, the adult ovariectomized rat being used and the dose divided into six injections in water over a period of 36 hr. The results reported in this paper make it probable that even the low potency of the trihydroxy form which remains after removal of the keto-hydroxy form is due to a conversion of the trihydroxy into the keto-hydroxy form in the organism, and that the presence of even an immature ovary facilitates this conversion. The problems as to which of the forms of keto-hydroxy oestrin (A or B) is secreted by the ovary in the organism under physiological conditions, whether they are interconvertible in the organism and under what conditions the organism is capable of converting the keto-hydroxy form into the trihydroxy form are of physiological significance. The occurrence in large amounts of the trihydroxy type in pregnancy urine in the human (10) makes the last process probable.

Summary

1. A crystalline oestrogenic substance has been isolated from human placenta. Evidence is presented for the identity of this substance with the active principle of the ether-insoluble compound previously demonstrated by Collip in a crude extract of human placenta and called by him "emmenin". The melting point of this substance is 274° C. (uncorrected). Analysis of two samples showed C, 74.8; H, 8.8% and C, 74.8; H, 8.9%. The crystal form has varied. The crystals are monoclinic, optically positive and show approximate refractive indices of α 1.54 and γ 1.68 and an inclined extinction of 7°. The solubility of the crystals in water at 100° C. is about 0.05%.

*The author has recently had the opportunity of making a direct comparison of the two substances in Dr. Butenandt's laboratory; emmenin (preparation No. 20) proved to be somewhat more active in the adult ovariectomized mouse than Butenandt's uncontaminated hormone-hydrate. Meanwhile a sample of the latter, kindly supplied by Dr. Butenandt, has been found at McGill University to be of the same order of potency as theolol and the emmenin crystals in the intact immature rat.

A mixed melting point of the compound with a sample of crystalline theelol gave no significant lowering of the melting point. The theelol melted at 276° C., the emmenin at 274° C., and a mixture of equal parts of both at 275° C. All values are uncorrected.

2. Emmenin was assayed on the adult ovariectomized white rat. The total dose was given in aqueous solution in six injections over 36 hr. The response of the animals varied according to their condition. A dose of about 15 γ was required to cause a squamous response in 50% of the animals used. The dose of theelol required for a similar response also varies but this substance was five to ten times more active than emmenin when tested on the same group of animals under the same conditions.

3. The dose of emmenin required for the immature intact rat of 21 days of age varies from 0.92 γ to 1.80 γ for a squamous response in 50% of the test animals when injected in six doses over 36 hr. The dose required when the emmenin is fed over a period of three days tends to be slightly lower than when injected as above. The dose of theelol for the immature intact rat is the same as that of emmenin both for feeding and injection experiments.

4. The dose of emmenin required for a 50% squamous response in the immature ovariectomized rat two to four days after ovariectomy was from 7 to 10 γ or about eight times that required for the immature normal animal of the same age. When tested on the immature ovariectomized animal the dose of theelol was about one and a half times that for the normal rat of the same age, that is about 2 to 3 γ . This increase is slight when compared to that taking place in the case of emmenin.

5. Transplantation of ovaries from 21-day-old rats into the spleen of immature ovariectomized rats causes the dose of emmenin necessary for a given response to fall from the level required for immature ovariectomized animals to that required for the immature intact animal. This fall in the dose occurs in the case of theelol also.

6. The activity of crystalline theelin when tested on the immature normal rat is nearly the same as that of emmenin. There is no change in sensitivity to theelin on ovariectomy of the immature animal. Transplantation of ovaries into the previously ovariectomized immature rat produced little change in sensitivity to theelin. The first dose of theelin sensitizes both ovariectomized and normal animals to a subsequent dose of the same material.

7. The theory advanced to account for these findings is that the immature ovary increases the efficiency of utilization of emmenin, and probably of theelol, by increasing the rate of conversion of these substances to a more active form of oestrogenic substance (probably some form of keto-hydroxy oestrin). This conversion can occur in the absence of the ovary, though much less effectively. It is not necessary that the ovary be producing cyclic phenomena for this conversion to take place.

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